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**Massachusetts Institute of Technology
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**Joint Program
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DOCTORAL DISSERTATION

*Evolutionary Patterns Within the Anthozoa (Phylum
Cnidaria) Reflected in Ribosomal Gene Sequences*

by

Ewann Agenbroad Berntson

June 1998

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Massachusetts Institute of Technology
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and

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Woods Hole, Massachusetts 02543

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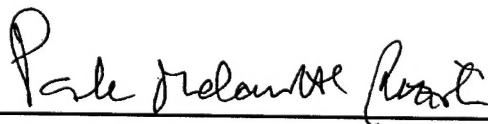
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**Evolutionary patterns within the Anthozoa (Phylum Cnidaria)
reflected in ribosomal gene sequences**

By

Ewann Agenbroad Berntson

B.Sc., University of Washington
(1991)

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
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DOCTOR OF PHILOSOPHY

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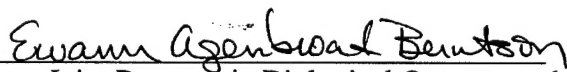
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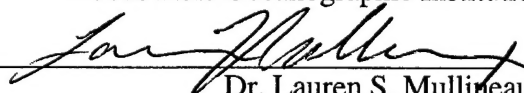
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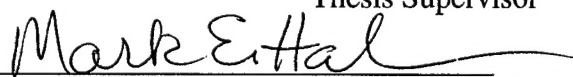
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Woods Hole Oceanographic Institution

*This thesis is dedicated
to the memory of
Jessica Wallace*

*You supported me through graduate school
in more ways than you can know.*

*You instilled in me an appreciation for the
truly important things in life.*

*You convinced me that I could make it
to the top of that mountain.
You were right, you know -- the view is
worth every step.*



Evolutionary patterns within the Anthozoa (Phylum Cnidaria) reflected in ribosomal gene sequences

by

Ewann Agenbroad Berntson

Submitted to the Department of Biological Oceanography in April, 1998
in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

Abstract

This thesis project assesses phylogenetic relationships within the phylum Cnidaria, at the subclass level within the Class Anthozoa, and at the ordinal level within the Subclass Octocorallia. Traditional cladistics using morphological data have resulted in disagreements over taxonomic relationships, primarily due to a paucity of morphological characters within the Anthozoa and ambiguity about the significance of any given character. I have used DNA sequence information to help resolve some of these issues. These phylogenetic studies contribute to the understanding of divergence within the class Anthozoa.

Museum collections of preserved flora and fauna historically used for morphological studies are now increasingly being utilized for addressing genetic questions. The extraction of DNA from ethanol-preserved specimens of recent origin is practiced routinely, but genetic analyses of long-preserved specimens have inherent difficulties due to the slow degradation of DNA. The goal of this study was to demonstrate the feasibility of isolating genomic DNA from museum specimens of octocorals and amplifying the nuclear 18S ribosomal RNA gene. The DNA sequence for the complete 18S rRNA gene can then be determined. Techniques were designed to solve several problems for obtaining genetic sequences from museum specimens. The DNA extractions of museum specimens yielded only small amounts of DNA of very low molecular weight, which limits the length of Polymerase Chain Reaction (PCR) products that can be generated with standard protocols. I was successful in producing PCR fragments from museum specimens by performing an extended tissue digestion on the archival specimens, running an initial PCR reaction, and then following with a reamplification of the original PCR product. The use of taxon-specific PCR primers decreased the risk of amplifying contaminant DNA rather than the target DNA in archival specimens. The combination of our modified extraction protocol and PCR reamplifications with taxon-specific PCR primers allowed me to generate 700- to 1800-basepair sequences from 16 specimens from three different museum collections that had been preserved for up to fifty years.

Taxonomic relationships within the corals and anemones (Phylum Cnidaria: Class Anthozoa) are based upon few morphological characters: colony morphology and the structure of the tentacles, gastric mesenteries, nematocysts, and skeletal axis. The significance of any given character is debatable, and there is little fossil record available for deriving evolutionary relationships. In this study I use complete sequences of 18S ribosomal DNA to examine subclass-level and ordinal-level organization within the Anthozoa. I investigate whether the traditional two-subclass system (Octocorallia, Hexacorallia) or the current three-subclass system (Octocorallia, Hexacorallia, Ceriantipatharia) is better supported by sequence information. I also examine the phylogenetic affinities of the anemone-like species *Dactylanthus antarcticus* and the putative antipatharian *Dendrobrachia paucispina*. Thirty-eight species were chosen to

maximize the representation of morphological diversity within the Anthozoa. Maximum likelihood techniques were employed in the analyses of these data, using relevant models of evolution for the 18S rRNA gene. I conclude that placing the orders Antipatharia and Ceriantharia into the Subclass Ceriantipatharia does not reflect the evolutionary history of these orders. The Order Antipatharia is closely related to the Order Zoanthidea within the Hexacorallia and the Order Ceriantharia appears to branch early within the Anthozoa, but the affinities of the Ceriantharia cannot be reliably established from these data. My data generally support the two-subclass system, although the Ceriantharia may constitute a third subclass on their own. The Order Corallimorpharia is likely polyphyletic, and its species are closely related to the Order Scleractinia. *Dactylanthus*, also within the Hexacorallia, is allied with the anemones in the Order Actiniaria, and their current ordinal-level designation does not appear to be justified. The genus *Dendrobrachia*, originally classified within the Order Antipatharia, is closer phylogenetically to the Subclass Octocorallia. The 18S rRNA gene may be insufficient for establishing concrete phylogenetic hypotheses concerning the specific relationships of the Corallimorpharia and the Ceriantharia, and the branching sequence for the orders within the Hexacorallia. The 18S rRNA gene has sufficient phylogenetic signal, however, to distinguish among the major groupings within the Class Anthozoa, and I can use this information to suggest relationships for several enigmatic taxa.

The Subclass Octocorallia (Phylum Cnidaria: Class Anthozoa) is comprised of the soft corals, gorgonian corals, and sea pens. The octocorals have relatively simple morphologies, and therefore few characters upon which to base taxonomic systems. Historically, the Subclass Octocorallia was divided into seven orders: Helioporacea (Coenothecalia), Protoalcyonaria, Stolonifera, Telestacea, Alcyonacea, Gorgonacea, and Pennatulacea. It has been argued that this arrangement exaggerates the amount of variability present among the species of the Octocorallia. The current taxonomy recognizes the two orders of Helioporacea (blue corals) and Pennatulacea (sea pens), and assembles the remaining species into a third order, Alcyonacea. The species within the Alcyonacea exhibit a gradual continuum of morphological forms, making it difficult to establish concrete divisions among them. The subordinal divisions within the Alcyonacea correspond loosely to the traditional ordinal divisions. In this study I address the validity of the historical ordinal divisions and the current subordinal divisions within the Subclass Octocorallia. I also explore the phylogenetic affinities of the species *Dendrobrachia paucispina*, which was originally classified in the Order Antipatharia (Subclass Ceriantipatharia). Polyp structure indicates a closer affinity between *Dendrobrachia* and the Subclass Octocorallia. I have determined the nuclear 18S rRNA sequences for 41 species of octocorals, and use these to construct a molecular phylogeny of the subclass. I utilize Maximum Likelihood techniques, employing a realistic model of evolution given these species and this data set. The most likely trees from these sequence data do not support the morphological taxonomy of the Octocorallia. The Order Pennatulacea is the most cohesive group within the subclass, but is not monophyletic. The most likely trees indicate three primary clades, one of which is undifferentiated and contains half of the species in this analysis. These data cannot distinguish among the branching order of these three clades. The morphological character of dimorphism (the presence of both autozooids and siphonozooids within a single colony) corresponds loosely with the topology of the most likely trees, and the monophyly of dimorphism cannot be rejected from these data. The species *Dendrobrachia paucispina* has a close affinity with the genera *Corallium* and *Paragorgia* (Alcyonacea: Scleraxonia), although its morphology suggests it is more similar to the genus *Chrysogorgia*. The genetic divergence found within genera is approximately equivalent to that found in other invertebrates, but the divergence found within families is greater in the octocorals than in other invertebrates. This difference may reflect the inappropriate inclusion of evolutionarily divergent genera within octocorallian families. This study is more thorough than other anthozoan molecular phylogenetic studies to date. I

have employed appropriate evolutionary models for maximum likelihood analyses, utilizing complete 18S rDNA sequences from the majority of families within the Octocorallia. Many of the relationships within the Octocorallia, however, remain ambiguous.

Thesis Supervisor: Dr. Lauren Mullineaux

Title: Associate Scientist, Woods Hole Oceanographic Institution

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As anyone who has ever conducted research knows, a project is rarely completed that didn't rely heavily upon help from others. Doctoral thesis projects are particularly good examples of this. I have been surrounded by numerous people who have generously donated their time, supplies, expertise, and specimens to help me, some doctoral student they had never heard of. Without them, my thesis project would have been woefully incomplete. And then there are those who have aided in the aspects of my life outside of science. They have given me support and friendship and chocolate regardless of how successful my analyses were that day. Their names won't appear on any of the papers I may publish, but their support was critical and very much appreciated.

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Chapter 1

Introduction

Knowledge of evolutionary systematics is fundamental to our understanding of the processes that influence the biological world. Life on this planet survives thanks to a complicated web of interactions involving species from microbes to mammals. The faunal diversity that exists today is the result of evolutionary processes acting over millions of years. The forces behind evolution, and the details of how species change, are not well-understood. Evolutionary changes cannot be observed directly on the time-scale of a human lifetime, so insight into these processes must be gained from other lines of evidence. The fossil record is invaluable for reconstructing morphological change. Not all species are readily fossilized, however, and often one must rely upon comparisons of existing morphological forms to hypothesize evolutionary relationships.

Evolutionary relationships among species can be reconstructed by comparing character traits, assuming that species sharing the greatest number of common traits are the closest relatives of one another. Traditionally, morphological characteristics were used to determine phylogenies because the genetic changes behind those morphological traits were inaccessible or unknown. With the advent of molecular techniques, we can use protein and DNA sequence information as additional characters to reconstruct phylogenetic relationships. DNA sequence information can be useful in determining relationships where morphological associations are ambiguous or contradictory and a fossil record is lacking. A combination of morphological and molecular information will provide the maximum number of characters upon which to base a taxonomic system.

This thesis examines phylogenetic relationships within the Class Anthozoa (Phylum Cnidaria). Cnidarians are some of the simplest of the invertebrates morphologically. They are diploblastic, possess a single body opening into a gastrovascular cavity, and have no specialized system for gas exchange, excretion, or circulation. The phylum is characterized by the presence of tentacles armed with stinging nematocysts. Many species exhibit an alternation of generations between a medusa stage

and a polyp stage. The species of the Class Anthozoa (anemones, corals, and sea pens) lack the medusa stage, and are exclusively polypoid.

An early taxonomic classification of the Anthozoa recognized two subclasses (e.g. Minchin et al. 1900, Deichmann 1936, Hyman 1940) (Fig. 1A). The Subclass Hexacorallia contained the stony corals (Scleractinia), anemones (Actiniaria), the black corals (Antipatharia), tube-anemones (Ceriantharia), and other anemone-like species (Zoanthidea and Corallimorpharia). The Subclass Octocorallia contained the blue corals (Helioporacea), soft corals (Alcyonacea), gorgonian corals (Gorgonacea), and sea pens (Pennatulacea). The subclass-level divisions were based primarily on the numbers of tentacles (eight in the Octocorallia vs. multiples of six in the Hexacorallia) and the number of divisions within the gastrovascular cavity, termed septa or mesenteries (eight unpaired in the Octocorallia vs. multiples of six paired in the Hexacorallia). The ordinal-level divisions within the Hexacorallia were based on the type of skeleton present, if any, and finer distinctions in the mesenteries and tentacles. The ordinal-level divisions within the Octocorallia were based on attributes such as colony structure, skeletal composition and arrangement, and the structure and location of extraskelatal calcium spicules (Wells & Hill 1956a).

The current taxonomic system, proposed as early as the 1890's (van Beneden 1897) but not adopted until the 1950's, recognizes three subclasses within the Anthozoa (Fig. 1B). The orders containing the black corals and the tube anemones were removed from the Hexacorallia and placed within their own Subclass, the Ceriantipatharia. This revision was based on two characters: the resemblance of the larval cerianthid (the cerinula) to the adult antipatharian polyp, and weak and indefinite mesentery musculature (Wells & Hill 1956b). The Subclass Octocorallia was also revised based on the hypothesis that the species of the Octocorallia were not sufficiently different from one another to warrant distinction at the ordinal level (Bayer 1981). The new octocorallian classification

Class Anthozoa
 Subclass Hexacorallia
 Order Actiniaria
 Order Zoanthidea
 Order Corallimorpharia
 Order Scleractinia
 Order Antipatharia
 Order Ceriantharia

 Subclass Octocorallia
 Order Helioporacea (=Coenothecalia)
 Order Protoalcyonaria
 Order Stolonifera
 Order Telestacea
 Order Alcyonacea
 Order Gorgonacea
 Order Pennatulacea

A

Class Anthozoa
 Subclass Hexacorallia
 Order Actiniaria
 Order Zoanthidea
 Order Corallimorpharia
 Order Scleractinia

 Subclass Ceriantipatharia
 Order Antipatharia
 Order Ceriantharia

 Subclass Octocorallia
 Order Helioporacea
 Order Alcyonacea
 Suborder Protoalcyonaria
 Suborder Stolonifera
 Suborder Alcyoniina
 Suborder Scleraxonia
 Suborder Holaxonia
 Order Pennatulacea

B

Fig. 1 **Historical and revised taxonomic divisions within the Class Anthozoa.**
A. Traditional classification, e.g. Minchin et al. (1900), Deichmann (1936), Hyman (1940).
B. Revised classifications, e.g. van Beneden (1897), Bayer (1981).

system retained the orders Helioporacea and Pennatulacea, and combined the remaining species into the Order Alcyonacea. The previous ordinal divisions were loosely maintained at the subordinal level within the Alcyonacea.

The Anthozoa have a relatively simple morphology, with few characters on which to base their taxonomy. The divisions within the Hexacorallia can include differences in the numbers of tentacles and mesenteries, as well as the presence or absence of a skeleton, but the octocorals all have the same number of tentacles and mesenteries, reducing the number of characters even further for defining groups within the subclass. Morphological characters are so few in the octocorals that sometimes an entire group is defined by a single character (Hickson 1930). The various taxonomic arrangements have arisen from differing interpretations of the significance of these characters (e.g. Broch & Horridge 1957 vs. Bayer 1993, concerning spicule morphology). The fossil record for the anthozoans is incomplete, because many of the species lack any solid skeleton which would be preserved more readily.

Genetic data can be particularly useful for investigating these types of taxonomic relationships. Many genes are found universally in all living species, and can therefore be compared directly. This is true for very few morphological characters. Different regions of DNA evolve at different rates as a result of functional constraints, so a genomic region can be selected to be appropriate for the scope of the evolutionary question addressed. New methods of analysis have recently become tractable for large data sets. Researchers can now use appropriate models of evolution in their analyses, reducing the error introduced into the analysis from incorrect assumptions about the way genes evolve.

In this thesis work, I used molecular phylogenetic methods to address questions of evolution within the Class Anthozoa. I sought to determine whether traditional morphological taxonomy or recent taxonomic revisions more accurately reflect the phylogeny of the class. Two specific questions pertaining to the Class Anthozoa have

arisen from conflicts in the interpretation of morphological data. The first question addresses the validity of the Subclass Ceriantipatharia within the Anthozoa. The placement of Ceriantharia and the Antipatharia in a separate subclass argues that these groups are of an evolutionary lineage separate from that of the other Anthozoa. A more fundamental aspect of this question is whether the ordinal divisions within the Hexacorallia and Ceriantipatharia, which are based exclusively on morphological differences, reflect the phylogeny of these species.

The second question concerns the degree of genetic divergence among the major groups within the subclass Octocorallia, and whether the morphologically recognized divisions correspond to genetic associations found. I examined whether genetic divergence indicates that the morphological groups should be distinguished at the ordinal or subordinal level. The separation of groups at the ordinal level indicates these groups are more distinct from each other than if they were separated at the subordinal level. The revised taxonomy (Bayer 1981) suggests a closer relationship among the alcyonacean suborders than among the orders Helioporacea, Alcyonacea, and Pennatulacea. Measures of sequence divergence will indicate the relative differences within and among the seven historically recognized orders. If divergence is lower among the suborders of the Alcyonacea than among the orders Helioporacea, Alcyonacea, and Pennatulacea, this would suggest that the Order Alcyonacea with the current subordinal divisions is a more accurate reflection of the evolutionary history of the Octocorallia.

This thesis project also examined the phylogenetic affinities of two taxa with intriguing morphologies. The first is the species *Dactylanthus antarcticus*, which is a member of the Order Ptychodactiaria within the Subclass Hexacorallia. The Ptychodactiaria are anemone-like, and were historically members of the Order Actiniaria (the anemones). Their musculature, nematocysts and mesenterial structure were subsequently deemed ancestral and unique from the other Actiniaria, and a new order was

created for them (Wells & Hill 1956c). The second species, *Dendrobrachia paucispina*, was originally classified with the Order Antipatharia (black corals) based on its skeletal axis. Recent examinations of polyp morphology suggest that *Dendrobrachia* is actually a member of the Octocorallia. Molecular information was utilized to clarify the phylogenetic affinities of these unusual species.

I acquired individual specimens or DNA from species representing all of the orders and the majority of the suborders and families in the Class Anthozoa in order to construct a molecular phylogeny of the class. Species from all orders and most suborders of the Hexacorallia were obtained for my analyses, as well as members of 22 of the 30 extant families of the Octocorallia. This sampling scheme was devised to include representatives from across the morphological breadth of the Class Anthozoa.

Specimens were collected by submersible from Fieberling Guyot in 1990 and Hawaiian seamounts in 1993 and 1996, and in coastal environments in the U.S., Bermuda, New Zealand, the Red Sea, and Panama. Sequences were determined from twelve alcohol-preserved specimens from the National Museum of Natural History, the Harvard Museum of Comparative Zoology, and the Bermuda Aquarium, Natural History Museum and Zoo.

Molecular Techniques

There are many steps in the progression from specimen to phylogenetic tree. Recent technological advances have improved the accessibility, quality and speed of many of the steps of phylogenetic analysis. Advances in analytical methods have accompanied the advances in techniques, providing a clearer understanding of molecular evolutionary processes in the natural world. As a result, the appearance of molecular phylogenies in the recent literature has increased geometrically.

Selection of a Genomic Region

Organismal phylogenies using molecular data are based on the assumption that a specific genomic region is representative of the evolutionary history of the entire genome. Sequence information from a single gene or portions of several different genes are evaluated because it is impractical to obtain the sequence of an entire genome. Each genomic region evolves at a specific rate determined by its structural and functional constraints, so a particular region must be selected that is appropriate for the phylogenetic question. A gene that evolves too slowly will yield insufficient genetic differences to indicate phylogenetic relationships among closely related taxa. A gene that evolves too rapidly will be difficult to align among species that are evolutionarily divergent. Genetic divergences greater than 30% indicate the sequences are essentially randomized, and therefore difficult to align with certainty (Hillis & Dixon 1991). Appropriate genomic regions must be chosen for addressing evolutionary relationships at the taxonomic levels under investigation.

Two basic types of genes that have been used for reconstructing molecular phylogenies are protein-coding genes and ribosomal genes. Protein-coding genes are constrained in the ways they can evolve because they must produce a functional protein. Ribosomal RNA genes, found in both the nucleus and mitochondria, do not produce a protein, but form part of an RNA and protein complex that builds other proteins. The RNA within these ribosomal complexes have extensive secondary structure, including regions that must maintain base-pairing with other portions of the RNA (stem regions), as well as regions which do not engage in base-pairing (loop regions) and are therefore less constrained. Because of these structural constraints, ribosomal RNA genes have domains that evolve at different rates, making them appropriate for phylogenetic analysis at a variety of taxonomic levels.

Nuclear ribosomal RNA genes are used frequently to address phylogenetic questions at a variety of taxonomic levels. In particular, the small-subunit 18S rRNA gene has proven useful for phylogenetic inquiries at the phylum level (e.g. Wainright et al. 1993), the ordinal level (e.g. Kelly-Borges et al. 1991, Hay et al. 1995) and the family level (e.g. Kuznedelov & Timoshkin 1993, Fitch et al. 1995). The questions I address in this thesis fall within this range, suggesting that the nuclear 18S rRNA gene is appropriate for this work. Although the 18S rRNA gene is fairly large (roughly 1800 basepairs in cnidarians), the availability of automated sequencers facilitates the sequencing process greatly. It is no longer prohibitive to generate many DNA sequences from genomic regions of this size.

Generating DNA Sequences

The determination of DNA sequences relies upon the generation of large quantities of DNA from the genomic region of interest. This was a time-consuming process in the past: the region of interest was identified through restriction digests and probing, the sample DNA was cloned into bacterial colonies, and then the colonies were grown and harvested to produce sufficient quantities of DNA to determine the sequence.

Undeniably, the most significant recent advance in molecular techniques as they relate to the generation of DNA sequences is the Polymerase Chain Reaction (PCR). PCR was first described in 1987 (Mullis & Faloona 1987). It is a process by which a particular genomic region can be copied exponentially by mimicking the cellular DNA replication cycle. DNA polymerase and individual nucleotides are added to the DNA template, along with small single-stranded (oligonucleotide) primers that anneal to the ends of the sequence to be determined. The regions flanking the gene of choice must therefore be known in order to design appropriate oligonucleotide primers. PCR is a highly effective procedure, producing a 200,000-fold increase in the target sequence in 20 cycles by starting with 1 µg

of total DNA (Mullis & Faloona 1987). The target sequence can be present in very minute quantities, which makes this procedure applicable for working with museum specimens, where tissue samples are limited and the majority of the DNA in the specimen may be damaged or degraded.

For my project, I performed PCR amplifications of the nuclear 18S rRNA gene, and I cloned the PCR fragments into bacterial colonies. There are three primary reasons for this approach. First, cloning gave me a permanent record of my PCR amplifications to which I could return if needed. Additionally, methods for determining the sequence of PCR products require large concentrations of those products. Generating large amounts of PCR product is routine from fresh or frozen specimens, but this was rarely the case with the museum specimens. Lastly, cloning the PCR fragments allowed me to determine the sequence for the entire fragment. Normally, the ends of the DNA template are 'lost' in the sequencing process, because sequencing primers must be slightly internal to the PCR primers to produce a clean DNA sequence. If the PCR fragment is cloned, however, one can begin the sequencing reaction within the bacterial vector, and read the entire PCR fragment.

I used the TA-cloning method (Invitrogen Corporation) in my protocol. TA-cloning takes advantage of a biochemical characteristic of the PCR reaction: *Taq* DNA polymerase adds an additional adenosine (A) nucleotide to the end of every synthesized DNA fragment. The bacterial vector is designed to have single thymidine (T) nucleotide on its ends which will pair-bond with the ends of the PCR fragments, and splice the DNA fragment into the vector. The vector with the PCR fragment is then transformed into bacterial cells, where it replicates along with the bacterial DNA replication system. The cells are grown on solid medium, and are harvested and their DNA extracted to be used as the template for the DNA sequencing reaction.

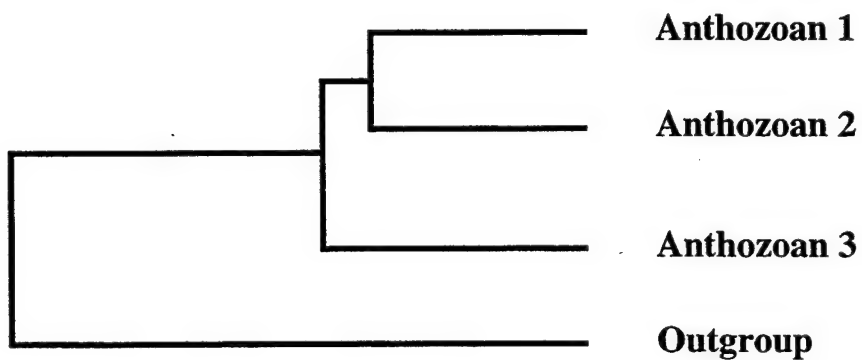
I utilized Licor automated sequencers for the bulk of this thesis project. I employed the dideoxy sequencing method (the Sanger method) for the sequencing reaction, with an infrared label on the sequencing primers. I performed six sequencing reactions for each specimen, with three primers along each strand of DNA for both strands. I determined the sequences for each strand in order to verify that the sequence was internally consistent. The images were analyzed using the BioImage program, which read the gels and assembled the individual reactions to form contiguous sequences.

Sequence Analysis

Once the sequences have been determined for a group of organisms, phylogenetic inferences can be made about those organisms based on the similarities and differences of their sequences. The first step in comparing sequences is to align them (Fig. 2A). An alignment can be thought of as an hypothesis of positional nucleotide homology. An alignment asserts that all nucleotides at a given position would share a common ancestral nucleotide. Computer programs are available that will align sequences, although many alignments can be constructed easily by eye. Sequence similarity is the primary basis by which sequences are aligned, but such alignments are subject to homoplasy (two nucleotides are the same at a given position through parallel evolution, not because they have a common ancestor). The use of stem and loop structure can be beneficial when aligning ribosomal sequences, incorporating knowledge of secondary structure. However an alignment is created, it is absolutely critical that the alignment be accurate before proceeding with further analyses. All phylogenetic analyses assume the alignment is correct, since the analyses intend to compare homologous characters. For this reason, any regions of the alignment that are ambiguous or difficult to align with certainty should be removed from the data set.

Anthozoan 1	C-CGCCTGTGGTGATTCATA
Anthozoan 2	CGCGCCTGTGATGATTCAGA
Anthozoan 3	AACGGCCGTGGTGATTCATA
Outgroup	GACGTCAGTCATCTTTAACT

A



B

Fig. 2 **Examples of a sequence alignment and phylogenetic tree**
A. Sample alignment of three theoretical species.
B. Hypothetical tree built from the above sequences.

The product of a phylogenetic analysis is a tree depicting relationships among the taxa included in the analyses (Fig. 2B). The individual taxa are found at the tips of the branches, and the branching pattern organizes the taxa into groups based on hypothesized evolutionary relationships. As one moves from the tips of the tree back to the base, one is moving back in time, retracing the development of the lineage of organisms. Each tree is considered an evolutionary hypothesis, based on the data presented and the algorithm used, representing proposed evolutionary relationships among the members of the tree. Therefore, trees of differing topologies are considered alternative evolutionary hypotheses.

The topological positions of taxa on a tree convey specific relationships among those taxa. A group of taxa that are united into a single cluster, joined together at a common base, is called a clade. Stated in a slightly different way, a clade constitutes a single point on the tree and all descendents. A group of taxa is considered monophyletic if all taxa in the group can be found in a single clade, with no other species included in that clade. A group of taxa is polyphyletic if members of that group can be found in different clades, with other species intermixed. A group is paraphyletic if it includes a common ancestor and some, but not all, of the descendents. Taxa that branch together with no other species between them are called sister taxa (e.g. Anthozoan 1 and Anthozoan 2 from Fig. 2B). Sister-taxa are presumed to be more closely related to each other than to any other taxon on the tree. Similarly, clades that branch together are called sister clades. A species that branches at the base of a clade is considered basal to that clade (e.g. Anthozoan 3 is basal to Anthozoans 1 and 2 in Fig. 2B). A basal taxon is considered to be ancestral to that clade, having diverged from it before the other members of that clade. Conversely, a species that branches nearest the tips of the tree (e.g. Anthozoan 1), is considered to be a derived species, having diverged from the lineage more recently. Trees can be constructed to depict the amount of dissimilarity (= genetic distance) among taxa by branch lengths.

The horizontal branch lengths between any two taxa on a distance tree is proportional to the genetic distance between those taxa.

Three primary algorithms are typically used for analyzing sequence data, each with its own theoretical basis. The most common method is parsimony, which operates under the familiar tenet that the simplest explanation is the most likely. As it applies to phylogenetic analyses, the tree that requires the least number of evolutionary steps to explain the relationships of character states among the taxa involved is considered the most parsimonious reconstruction of those taxa. Parsimony analyses only consider similarities, and thus they are the most susceptible to the effects of convergent or parallel evolution.

A second suite of analyses are the distance methods. Distance analyses convert all information on sequence differences between two taxa to a single number, representative of the differences between those taxa. Various evolutionary models can be built into distance calculations, taking into consideration aspects such as variations in substitution rate and base frequencies. The actual sequence information is not retained once the distance calculation is made, which results in a loss of information. But the algorithm is relatively fast as a result, making it particularly useful for large data sets. Distance methods are not as accurate in finding the correct tree, however, if there are many short branches.

Maximum Likelihood (ML) methods have been developed most recently for practical use with sequence data sets. ML constructs phylogenetic trees that are the most likely given the sequence data being analyzed and the evolutionary model employed (Swofford et al. 1996, Huelsenbeck & Crandall 1997). The analysis produces a statistic for each tree, the maximum likelihood score (Lscore) that is the probability of that tree given the data set and the model. This score can be used to compare two different trees (i.e. two evolutionary hypotheses) statistically.

Maximum likelihood techniques allow one to tailor the evolutionary model used specifically for the analysis of each individual data set. This is especially important when

working with large data sets, where the error introduced from using an incorrect method of analysis can overwhelm the phylogenetic information in the data set (DeSalle et al. 1994, Rzhetsky & Nei 1995).

The types of parameters ML can use in its evolutionary models include unequal base frequencies, base-specific rates of change (i.e. A to C, G to T, etc.), a proportion of sites that are invariant, and a variable substitution rate among the remaining sites. One can choose between having one, two, or six different rates of substitution. One rate of substitution effectively gives all sites the same mutational rate. Two substitutional rates allows the differentiation between transitions (A to G, C to T) and transversions (A to C, G to T). Six rates of substitution allows a rate of substitution for each possible base change (A to C, A to G, A to T, etc.). Further heterogeneity in substitutional rate may be present in the data, especially in ribosomal sequences. The gamma distribution is often used to model this heterogeneity: a few sites have a high substitutional rate (potentially multiple substitutions at a single site), and the majority of sites have a very low substitutional rate. The exact shape of the gamma distribution is determined by the gamma parameter, α , which is the inverse of the coefficient of variation for the distribution. The most complex ML model incorporates all of the parameters just described (unequal base frequencies, six rates of substitution, a proportion of invariant sites, and rate heterogeneity modeled with a gamma distribution). This model is referred to as a General Time-Reversible model (GTR) with substitutional rate heterogeneity (discussed in depth in Swofford et al. 1996).

Maximum likelihood calculations will indicate which evolutionary parameters should be incorporated when analyzing a particular data set. The most accurate evolutionary model will always be the GTR model with rate heterogeneity, described above, but it is also the most complex (i.e. has the most parameters to calculate). It is not always necessary to use the most complex model. The simpler models incorporate fewer parameters (i.e. unequal base frequencies, proportion of invariant sites, etc.) and are

therefore less computer-intensive, and will run more quickly than the more complex models. A simpler method will also produce lower variance. A simpler model, however, may not be sufficiently accurate. In order to determine which model is the simplest for a given data set without sacrificing accuracy, a tree is generated from that data set with any of the standard models (parsimony, distance, etc.). The Lscores are then calculated for that tree using a number of different evolutionary models, and compared using the Likelihood Ratio Test (LRT) (Swofford et al. 1996, Huelsenbeck & Rannala 1997). The LRT will determine if a simpler model can be used, or if a more complex model will give significantly better results. Once the appropriate evolutionary model has been determined, the same initial tree is used to calculate the correct parameters for the model chosen based on the particular data set. Those parameters are then used in an ML analysis of the data.

Once the parameters have been chosen for a given data set, a search for the most likely tree is performed. The most thorough method of searching is termed an exhaustive search, in which all possible branching combinations of taxa are tried and evaluated. The number of possible trees increases geometrically with the number of taxa, however. Only one tree is possible with three taxa, but four taxa can yield 3 trees, five taxa yield 15 possible trees, six taxa generate 105 possible trees, etc. (Swofford 1991). A data set like those used for chapters 2 and 3 include over 50 taxa, which yield well over 3×10^{74} possible trees. Computationally, this is very intensive. As a result, I have conducted heuristic searches of my data sets.

Heuristic searches are must faster than exhaustive searches, but they are not guaranteed to find the optimal tree. Heuristic searches are conducted by constructing an initial tree, and then swapping branches on that tree in an attempt to improve the tree. The search will settle on the best tree possible given the original starting tree. This type of searching runs the risk of finding a 'locally optimal' tree rather than a 'globally optimal' tree. The best way to increase the chances of finding the globally optimal tree is to perform

multiple heuristic searches, with different starting trees. This is what I have done for the bulk of my data analyses.

Once the most likely tree (or trees) have been determined, manipulations of those trees can yield valuable information about the strengths and weaknesses of the phylogenetic signal. The 'most likely' tree is indeed most likely, but it may not be statistically worse than a tree with an alternate topology. I used the computer program PAUP* (Swofford 1996 betatest version) in conjunction with the tree-building program MacClade (Maddison & Maddison 1992) throughout my thesis work to build phylogenetic trees and test their topologies. The simplest way to test alternate evolutionary hypotheses is to construct trees in MacClade, and then measure the Lscores of both trees in PAUP* using the appropriate evolutionary model. The Kishino-Hasegawa test (KH Test) (Kishino & Hasegawa 1989) within PAUP* is a two-tailed statistical test to determine if one tree is statistically less likely than the other.

PAUP* offers two methods that I used for testing the topological support for phylogenetic trees. The first is a search where a portion of a tree is constrained. I used constrained searches to test the branching order for the primary clades in my trees: I constrained the topology within each of the clades, then performed a search and kept every tree that was constructed. The KH Test can then determine if one branching pattern is better than the others, and if alternate branching patterns are significantly less likely than the best.

The backbone search is another type of constraint, and will determine where a given taxon can branch on a tree without significantly reducing the likelihood of the tree. Some taxa will fall in a single position on the most likely tree, but the tree may not be much less likely if the taxa are placed in a different position. The backbone search is a simple way to test all possible positions for a given taxon without creating and testing each tree individually. The entire tree is constrained for this search as a backbone, minus the taxon

being tested. An exhaustive search is conducted and all trees are saved. The KH Test will show which branching positions are not significantly less likely than the optimal position.

Specific Issues for this Project

Museum Specimens

Museum collections contain a wealth of information for both morphologists and geneticists (Thomas 1994). These collections have been assembled over the last few centuries, and contain species that are rare, not easily accessible, or even extinct. Museum collections constitute an invaluable resource for countless research questions.

The use of museum specimens for genetic analyses has inherent difficulties, relating primarily to the degraded nature of archival DNA. Despite fixation, DNA continues to degrade over time. Archival DNA is damaged primarily by oxidation and hydrolysis (Lindahl 1993). Oxidation of the pyrimidines (cytosine and thymidine) is the primary complication for PCR reactions (Hoss et al. 1996). As a result of this damage, only small stretches of DNA can be PCR amplified from most museum specimens.

The traditional method of preservation for museum specimens is formalin. Formalin preserves tissue by crosslinking DNA and proteins to themselves and each other (Fox et al. 1985) which is highly effective for the preservation of archival specimens. The same cross-linking creates hydroxymethyl groups on the DNA, which interfere with the PCR process. Additionally, much of the DNA remains tightly bound to proteins and is lost through the DNA extraction procedure (Shedlock et al. 1997). PCR and sequencing reactions can be successfully performed on formalin-fixed tissues, but they require special handling and only small regions of DNA (50-500 bp) can be amplified (France & Kocher 1996, Shedlock et al. 1997).

Octocorals have traditionally been preserved in ethanol, rather than formalin, because the formalin dissolves the calcium spicules which are used in species identification.

The absence of formalin increases the feasibility of determining longer sequences from museum octocoral specimens. DNA extractions on recently ethanol-preserved specimens are relatively straightforward (Kocher 1992), and I was able to extract and PCR-amplify DNA from specimens that had been preserved up to 50 years. Chapter 2 describes the protocol I used with the alcohol-preserved specimens.

Most of the museum specimens for which I attempted PCR reactions yielded no PCR product after an initial PCR reaction. In order to generate sufficient PCR product for the sequencing process, I had to perform PCR reamplifications for several specimens: an initial PCR amplification was performed, and an aliquot of the first reaction was used as the template for a second round of PCR. The negative control from the initial PCR reaction was always included as the template for a negative control for the second PCR reaction. Even using PCR reamplifications, I was only able to amplify the entire 18S gene in one piece for one specimen. Several specimens were amplified in two or three pieces to generate the entire 18S sequence.

The degraded nature of the archival DNA, as well as the multiple rounds of PCR performed on each specimen, increases the likelihood that contaminant DNA rather than the target DNA will be amplified. PCR preferentially amplifies molecules of DNA that are intact, so a contaminant of recent origin (i.e. introduced to the specimen after it was preserved) may be more likely amplified than the older, sample DNA. One way to increase the chances of amplifying the target DNA is to design taxon-specific PCR primers. I designed a suite of primers that matched the DNA of octocoral specimens for which I knew the sequences, but not the DNA of fungi, symbionts, or other phyla of organisms that might be found as contaminants. All sequences were compared to the GenBank database after they were determined, to verify that they were most similar to other cnidarians and not a potential non-Cnidarian contaminant.

Pseudogenes

An additional difficulty I encountered during this thesis work, probably relating to the degraded nature of the museum specimens, was the amplification of pseudogenes rather than the target 18S gene. Normally, the multiple copies of the ribosomal genes found within each cell are assumed to be identical, resulting theoretically from concerted evolution (Hillis & Dixon 1991). Pseudogenes are copies of ribosomal genes that are not identical to the majority, and are also not transcribed. They are similar to the functional ribosomal gene but are not functional themselves, and as a result, evolve neutrally. Pseudogenes have been found in several invertebrate taxa, and have diverged from the functional copy of the ribosomal gene. Pseudogenes found in the flatworm *Dugesia* were approximately 8% different from the transcribed copies of the 18S gene, which approaches the divergence found between families for this group (Carranza et al. 1996). This level of divergence corresponds to the divergence found between classes and subclasses of anthozoans (discussed in Chapter 3). Seven of the sequences that I determined from museum specimens were most likely sequences of pseudogenes rather than the functional nuclear 18S rRNA gene. All of these putative pseudogenes clustered together in my analyses, and they branched most closely with the Order Scleractinia (Subclass Hexacorallia) rather than the Octocorallia to which they belonged. These specimens were excluded from my analyses.

I encountered pseudogene sequences in determining the sequence for *Dendrobrachia paucispina*. The specimen had been alcohol-preserved for eight years, and two PCR fragments (with a 1065-bp overlap) were required to construct the entire 18S sequence. The sequences were determined for four clones of each fragment. The clones for the first half were all identical, but the clones for the second half were all different. Each of the four clones were verified as cnidarian by a search of the GenBank database, but only one of the sequences was identical to the first half of the gene, based on an

overlapping region of the two sequences. I used the clone that was identical to the first half to complete the *Dendrobrachia* sequence, and hypothesized that the other sequences were from pseudogenes. The complete sequence placed *Dendrobrachia* within the Octocorallia, as expected from morphology, and not with the cluster of pseudogenes near the Scleractinia. I am therefore relatively confident that the sequence for *Dendrobrachia* is real, and not an artifact. Sequences from additional specimens of *Dendrobrachia* would be necessary to verify the validity of the sequence I have generated.

Previous Molecular Studies

Molecular studies of the Anthozoa are few, but they have given us some insight into the questions I address with this thesis. These studies have examined a variety of ribosomal genes, including the mitochondrial 16S rRNA gene (France et al. 1996), the nuclear 28S rRNA gene (Chen et al. 1995), and the nuclear 18S gene (Song & Won 1997). They examine the subclass divisions within the Anthozoa and the ordinal divisions within the Hexacorallia and the Octocorallia, but do not address the phylogenetic affinities of *Dactylanthus* or *Dendrobrachia*. All of these studies used general parsimony or distance methods rather than the more accurate maximum likelihood methods. None of these studies have the breadth of taxonomic sampling that this study does, and all of them use partial gene sequences. But these studies offer both support for, and alternate hypotheses to, what I have found in my thesis work.

Mitochondrial 16S (France et al. 1996) and nuclear 18S (Song & Won 1997) sequence information have both indicated that the Ceriantharia and the Antipatharia, united within the Subclass Ceriantipatharia, are genetically divergent from one another. The nuclear 18S analyses indicated that the Ceriantharia are ancestral to all other Anthozoa, and the Antipatharia have affinities within the Hexacorallia. Mitochondrial DNA supported that the Antipatharia branch within the Hexacorallia, but indicated that the Ceriantharia were

ancestral to the Hexacorallia rather than the entire Anthozoa. Neither study had complete taxonomic representation of the orders in the Hexacorallia to determine the specific affinities of the Antipatharia. My thesis work included representatives of all of the orders within the Hexacorallia, and therefore I could generate a more complete analysis of the subclass divisions within the Anthozoa.

The ordinal-level divisions within the Hexacorallia are supported by mitochondrial rDNA (France et al. 1996) and 18S rDNA (Song & Won 1997), but not entirely by 28S rDNA (Chen et al. 1995). The Scleractinia were monophyletic in Chen et al.'s analyses, but both the Actiniaria and Corallimorpharia were polyphyletic. Chen et al. used very short sequences, however, which may have affected the results they found. I have included over 1600 bp of sequence information spanning the entire 18S rRNA gene for my analyses.

Molecular information is not as available for addressing the divisions within the Octocorallia. The France et al. study (1996) is the most thorough so far, including representatives from the Pennatulacea, and 16 species from 10 families within the Alcyonacea. Mitochondrial DNA sequences do not indicate a correlation between phylogenetic structure and traditional taxonomy within the Octocorallia. The analyses of Song and Won (1997) did not disagree with the current taxonomy, but they include only a single pennatulacean and four species from two families of the Alcyonacea. Both studies did, however, find the Pennatulacea to be ancestral within the Octocorallia. My thesis project continues this work, including representatives of 22 families within the Order Alcyonacea, a representative of the Order Helioporacea, and eight representatives of the Order Pennatulacea.

Summary

The goal of this thesis project was to build the most complete molecular phylogeny of the Anthozoa to date. A number of specific questions were addressed with this work, relating to the taxonomic divisions within the Class Anthozoa, and within the subclasses Hexacorallia, Octocorallia, and Ceriantipatharia:

- Does genetic information support division of the Class Anthozoa into two subclasses or three? Is the association of the Ceriantharia and the Antipatharia in the Subclass Ceriantipatharia valid, based on evolutionary history?
- Does genetic sequence information support the division of the Subclass Octocorallia into three orders or seven? Do the traditional morphological divisions correspond to the phylogenetic divisions I find?
- Are the Ptychodactiaria deserving of ordinal distinction separate from the Actiniaria within the Hexacorallia?
- Is *Dendrobrachia* affiliated with the Antipatharia, with which it was previously classified? Or is it more closely related to the Octocorallia, as indicated by polyp morphology?
- Which morphological characters appear to provide evolutionary information? Which characters clearly do not?

In order to address these questions, I determined the nuclear 18S rDNA sequences from 58 species across the Class Anthozoa. I included 12 previously sequenced anthozoans, available from the GenBank database, as well. This sampling scheme gave me representatives from across the morphological breadth of the entire class. I used the complete 18S sequence, and conducted my phylogenetic analyses using maximum likelihood techniques. ML allowed me to tailor the evolutionary model I used to be

appropriate for this particular gene and this group of species. I also used ML techniques to compare alternate evolutionary hypotheses statistically, and thereby test hypotheses suggested in previous molecular studies.

This thesis project further developed techniques for utilizing ethanol-preserved museum specimens for DNA sequence analyses. DNA extraction and PCR amplification of recently-preserved (i.e. 1-2 years) specimens is routinely practiced in many laboratories, but this is not true for long-preserved (i.e. 10-50 years) specimens. DNA can be successfully extracted and PCR amplified from recently preserved specimens, but archival specimens require special handling and protocols. Chapter 2 describes the techniques that were successful for determining DNA sequences from museum specimens of octocorals.

Knowledge of how species evolve is essential to our understanding of the natural world. The species that are on the Earth today are the direct result of millions of years of evolutionary change, and that evolution is continuing. The taxonomic divisions within the Anthozoa are difficult to determine using only morphological characters, as anthozoans are some of the simplest invertebrate species. The addition of molecular characters gives us a common character to compare across all species within the class, and which can also be compared to other invertebrate taxa.

Anthozoans are a very important group of taxa from an ecological view. Species of anthozoans can be found everywhere throughout the ocean, from the shallow intertidal to the deep sea. Species in the Order Scleractinia create the foundation for the coral reef ecosystem, which is highly complex and very fragile. Members of both the Subclass Octocorallia and Hexacorallia are also prominent on reef ecosystems. A better understanding of how these species are related to each other and how they change over time may help us conserve these delicate environments for years to come.

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Chapter 2

Generating DNA sequence information from museum collections of octocoral specimens (Phylum Cnidaria: Class Anthozoa)

Abstract

Museum collections of preserved flora and fauna historically used for morphological studies are now increasingly being utilized for addressing genetic questions. The extraction of DNA from ethanol-preserved specimens of recent origin is practiced routinely, but genetic analyses of long-preserved specimens have inherent difficulties due to the slow degradation of DNA. The goal of this study was to demonstrate the feasibility of isolating genomic DNA from museum specimens of octocorals with subsequent amplification of the 18S rRNA gene. Techniques were designed to solve several problems for obtaining genetic sequences from museum specimens. The DNA extractions of museum specimens yielded only small amounts of DNA of very low molecular weight, which limits the length of Polymerase Chain Reaction (PCR) products that can be generated with standard protocols. I was successful in producing PCR fragments from museum specimens by performing an extended tissue digestion on the archival specimens, running an initial PCR reaction, and then following with a reamplification of the original PCR product. The use of taxon-specific PCR primers decreased the risk of amplifying contaminant DNA rather than the target DNA in archival specimens. The combination of our modified extraction protocol and PCR reamplifications with taxon-specific PCR primers allowed me to generate 700- to 1800-basepair sequences from 16 specimens from three different museum collections that had been preserved for up to fifty years.

Introduction

Scientists and lay persons have contributed specimens to museum collections world-wide for well over one hundred years, creating a highly useful resource for investigators today. Many of these specimens have been collected during expeditions, while others have been collected incidentally in the course of other pursuits. These preserved specimens can be used to address a variety of questions.

Museum collections have been, and continue to be, used extensively for morphological studies, but development of techniques utilizing the Polymerase Chain Reaction (PCR) have increased the potential value of museum collections for investigating genetic questions. The molecular analysis of archival specimens can lend insight not only into evolutionary or phylogenetic investigations among taxa, but also for questions of gene flow within species. Changes in allelic frequencies can be traced through time or across geographic areas (Kocher 1992, Thomas 1994). Such allelic changes may indicate levels of genetic variation within species, changes in genetic variation over time, hybridization events between species, or range expansions or contractions. Museum specimens can also supply genetic information for species that have recently become extinct or are currently endangered (Thomas 1994).

Extracted DNA from fresh or frozen tissue is of considerably higher molecular weight compared to that from preserved specimens. Therefore, genetic analyses are easier to conduct using fresh or frozen tissue; however, the use of preserved specimens is preferable in many cases. Collection of fresh samples from rare species or those with small population sizes is usually inappropriate or impossible. Many species live in remote habitats that are highly inaccessible, and require great expense for sample collection. The use of existing collections in these cases are preferable to the acquisition of new specimens (France and Kocher 1996).

Museum specimens have been subjected to variable handling and preservation techniques. The type of preservative used, the speed with which a sample is preserved, and the subsequent methods by which a sample is handled will have a large effect on the resulting condition of the DNA. The handling will determine, in part, the utility of a specimen for a given research question. Cryogenic preservation is often preferable for genetic studies as liquid nitrogen maximizes DNA extraction yields. Liquid nitrogen, however, was not available historically, and currently is not always available in all localities or field conditions. A number of alternative preservation techniques have been employed, including drying, the use of various alcohols, formaldehyde, mannitol-sucrose buffer with EDTA, and guanidine hydrochloride (Dessauer et al. 1996).

Most museum specimens have been dried or preserved in formalin and the latter transferred to ethanol for long-term storage. Formalin preservation cross-links molecules of proteins and nucleic acids to themselves and to each other (Fox et al. 1985). Hydroxymethyl groups are also formed on the DNA molecules (Chang and Loew 1994). The tight crosslinking of DNA to proteins is problematic for DNA extraction procedures, as much of the DNA is thereby lost into the organic phase of the phenol extraction procedure (Shedlock et al. 1997). The methylation can interfere with PCR replication by impeding primer annealing as well as derailing the DNA polymerase during the extension phase (Karlsen et al. 1994). Protocols have been developed for successfully obtaining sequence from formalin-preserved specimens, in which the preserved specimen is soaked in buffer followed by extended periods of digestion with proteinase K which permits the DNA to dissociate from the protein complexes. PCR replication of formalin-fixed tissues remains difficult, however, and PCR amplification is only possible for short (i.e. 50-300 bp, and rarely 500-600 bp) stretches of formalin-fixed DNA (France and Kocher 1996; Shedlock et al. 1997).

Museum collections of octocoral specimens are unusual in that they are often preserved initially in ethanol rather than formalin, because formalin dissolves the calcareous spicules that are used in species identification. The decreased likelihood of the use of formalin in the preservation and storage of octocorals increases the feasibility of the DNA isolation process greatly. The extraction of DNA from recently (i.e. a few months or years) ethanol-preserved specimens is now practiced routinely in a number of laboratories (e.g. Smith et al. 1987; Kawasaki 1990; Meyer et al. 1990; Wheeler et al. 1993). Unlike formalin, ethanol does not cross-link proteins, but it is less efficient as a tissue preservative. Several studies have documented the degradation of DNA over time in ethanol-preserved specimens (e.g. Post et al. 1993; Flournoy et al. 1996). Specimens stored in ethanol at lower temperatures tend to preserve better (Post et al. 1993; Hoss et al. 1996), and PCR amplifications of shorter lengths of DNA are typically more successful than longer lengths in older samples. Very few studies have been published based on obtaining genetic information from specimens preserved in ethanol for longer than a few years.

Genetic analyses of long-preserved specimens have inherent difficulties, due to the slow degradation of DNA in the presence of a fixative. The DNA damage that occurs in preserved samples is primarily due to oxidation and hydrolysis (Lindahl 1993). The damage that is the most detrimental to the performance of genetic analyses includes modifications to the pyrimidines, cross-linking between molecules, and missing bases (Paabo 1989). Oxidative modifications to purines, however, do not seem to affect the ability to generate sequences from preserved materials (Hoss et al. 1996). These types of modifications will particularly hinder the successful direct cloning of ancient DNA, because damaged DNA will not be copied by the bacterial replicative process. In the event that cloning is successful, however, often the bacterial replication process will repair any damage it perceives, thereby introducing error into the sequence (Paabo et al. 1989).

PCR is also hindered by damaged DNA, but offers two unique advantages when working with ancient DNA. First, the PCR will preferentially amplify the DNA molecules that remain intact, because the DNA polymerase will move more slowly over damaged regions (Paabo et al. 1989). Additionally, any modified DNA strand that is replicated will be unlikely to have a sizable effect on the resulting genetic sequence unless it is replicated early in the PCR process. If a damaged strand does get replicated early on, however, the genetic sequence that results will be influenced more substantially by those damaged regions.

Another difficulty in using archival specimens for genetic analysis is the increased possibility that contaminating DNA will amplify rather than the target DNA, which may be degraded and in low concentrations (Paabo 1990). The amplification of contaminating DNA is rarely a significant problem when using fresh or frozen tissue, as fresh DNA extractions yield large quantities of non-degraded DNA, and concentration of the target DNA usually far exceeds any that of any contaminating DNA. Museum specimens may contain trace DNA not only from symbionts or epibionts, fungi and bacteria, which can be introduced both before or after preservation. Contaminating DNA of modern origin will likely be less degraded than the ancient target DNA, and may be more readily amplified in the PCR process. Even if the contaminating DNA is degraded, there is still a chance it will be amplified instead of, or along with, the target DNA. Extreme caution must be taken when working with archival specimens to avoid amplifying contaminant DNA. One method which can be effective in eliminating the amplification of contaminating DNA is the use of taxon-specific PCR primers. Specific primers are designed using regions of the target DNA that are characteristic of the target but not of potential contaminants. A well-designed specific primer will preferentially amplify the target DNA to the exclusion of extraneous DNA.

Complete sequences for the 18S rRNA gene were desired for a companion study on the phylogeny of the Class Anthozoa, and in particular the Subclass Octocorallia (Berntson et al. in prep). Because many of the specimens for this study had been preserved for up to fifty years and their DNA was potentially highly degraded, standard DNA extraction and PCR techniques proved unsuccessful. Museum collections were essential to this phylogeny project as many octocoral species, including entire families, are found exclusively in the deep sea. Fresh specimens of these species are accessible only by submersibles or dredging, and it was not feasible to collect new specimens of all the necessary species.

There were two primary goals of this study: 1) to develop a technique for the isolation of DNA from preserved material, yielding as large quantities of nondegraded DNA as possible, and 2) to PCR amplify the 18S rRNA gene from the isolated genomic DNA in fragments greater than 500 bp in length, with the ultimate purpose of determining the DNA sequence of the complete 18S rRNA gene from the preserved octocoral specimens. The use of a modified DNA extraction protocol combined with taxon-specific PCR primers described herein have allowed us to accomplish these goals.

Methods

Specimens

Specimens for this study were acquired from Dr. Frederick Bayer of the National Museum of Natural History, Dr. Wolfgang Sterrer of the Bermuda Aquarium, Natural History Museum and Zoo, and Ardis Johnston of the Harvard Museum for Comparative Zoology. These specimens had been stored in ethanol for periods ranging from two to 50 years.

DNA Extraction Protocol

The extraction protocols I used was similar to those described by Coffroth et al. (1992) and Winnepenninckx et al. (1993). My deviations from their protocols consisted primarily of a lower temperature and longer duration for the proteinase K digestion. The extraction buffer included 1.4M NaCl, 0.02M EDTA, 0.1M Tris-HCl (pH 8.0), 2% cetyltrimethylammonium bromide (CTAB) (Sigma Chemical Co.), and 0.2% beta-mercaptoethanol (Sigma Chemical Co.). This buffer is particularly effective at removing polysaccharides that are abundant in coral tissues, and which can interfere with DNA extraction. General CTAB-based protocols have been described for use with a variety of invertebrate taxa, including algae, molluscs, ctenophores, and brachiopods (Karl and Bailiff 1989; Shivji, Rogers et al. 1992; Winnepenninckx, Backeljau et al. 1993).

Five to ten polyps of the ethanol-preserved octocorals were placed on ice in two to ten mls 2X CTAB buffer for two to 24 hours, with the buffer replaced several times during this period. The buffer was removed, and the tissue minced finely with a razor blade and placed in a 1.5-ml eppendorf tube with 300 µl of 2X CTAB buffer. A plastic dounce was employed to further shear the tissue, and an additional 300 µl of 2X CTAB was added. The samples were placed at 55°C and digested with 5 µl of proteinase K (at 20 mg/ml) for approximately 24 hours, with periodic agitation. Another 5 µl of proteinase K was added, and the tissues continued to digest for an additional eight to twelve hours. The tissues were extracted once with an equal volume of 24:1 chloroform:isoamyl alcohol, and precipitated in two volumes of cold 95% ethanol at -20°C overnight. The tubes were centrifuged at 10,000xg for 30 minutes, and the ethanol was removed. The pellets were washed with 500 µl cold 70% ethanol, and the tubes were centrifuged at 7,000xg for 15 minutes. The ethanol was removed, and the pellets dried at room temperature. The pellets were resuspended in 50 µl of TE buffer and placed at 4°C for three to four hours before visualization on an agarose gel.

DNA Amplification Protocols

Pipet tips with a filter barrier were used throughout this process to guard against contamination of the reactions. Negative controls were included during the DNA extractions and PCR reactions to detect contamination if it did occur. Each extracted DNA sample was diluted 1:10 in TE buffer (10 mM Tris-HCl--pH 8.0, 1 mM EDTA--pH 8.0), and 2 μ l of that dilution was used in a 50- μ l PCR reaction. Modified versions of the universal eukaryotic primers A and B from Medlin et al. (1988) (Table 1) were used in the initial DNA amplifications of the 18S rRNA gene. These primers amplify the entire anthozoan nuclear 18S rRNA gene, a fragment which we found to be approximately 1800 bp in length in anthozoans. Thirty-five cycles of PCR were carried out in a Perkin-Elmer Thermocycler 480. The DNA was denatured at 94°C for 45 seconds, the primers and template were annealed at 55°C for one minute, and the original DNA strand was extended at 72°C for 90 seconds. These cycles were followed by a five-minute extension at 72°C. The product was run on a 1% agarose gel. If there was a visible product, that product was prepared for TA-cloning.

For those specimens with no visible product, a second PCR reaction was conducted using 1 μ l of product from the initial PCR reaction as the template. The amplified negative control (no DNA was added) from the initial PCR reaction was included in the second reaction as well, using 1 μ l of the original negative control as template. The primers used for the second PCR reaction were chosen from those listed in Table 1, with at least one primer falling internally to the initial A and B primers. The internal primers were selected from a combination of universal eukaryotic primers and a set of octocoral-specific primers that were designed by us (Table 1). The octocoral-specific primers were designed to amplify anthozoan DNA, but not DNA from potential contaminants. The octocoral-specific primers were designed from alignments of Genbank sequences of actinarians (*Anemonia sulcata*, Genbank accession #X53498, and *Anthopleura kurogane*, accession #Z21671),

Table 1 **PCR primers used for primary and secondary amplifications of DNA from octocoral specimens. The octocoral-specific primers were designed for phylogenetic studies of the Subclass Octocorallia (described in Berntson et al. in prep.). Primer numbers refer to position in prokaryotic small-subunit ribosomal DNA.**

Primer	Specificity	Sequence	Reference
A (F)*	Univ. Euk.	5' AACCTGGTTGATCCTGCCAGT 3'	Medlin et al. (1988)
B (R)**	Univ. Euk.	5' TGATCCTTCTGCAGGTTCACTAC 3'	Medlin et al. (1988)
514F	Univ. Euk.	5' GTGCCAGCMGCCCGCG 3'	Elwood et al. (1985)
536R	Univ. Euk.	5' WATTACCGCGGCKGCTG 3'	Lane et al. (1985)
1055F	Univ. Euk.	5' GGTGGTGCATGGCCG 3'	Elwood et al. (1985)
1055R	Univ. Euk.	5' CGGCCATGCACCACC 3'	Elwood et al. (1985)
373F	Univ. Euk.	5' GATTCCGGAGAGGGAGCCT 3'	Weekers et al. (1994)
1200R	Univ. Euk.	5' GGGCATCACAGACCTG 3'	Weekers et al. (1994)
705F	Octocoral	5' GGTACAGCCGTAAGGTTT 3'	present study
705R	Octocoral	5' CATACCTTCGGCTGACC 3'	present study
900F	Octocoral	5' GTTGGTTTTTGAACCGAAG 3'	present study
900R	Euk.	5' CTCGGTTCTAGAAACCAAC 3'	present study
1560R	Octocoral	5' GGTGAAGGAGTTACTCGATG 3'	present study

*F sequences prime in the 5' -> 3' direction

**R sequences prime in the 3' -> 5' direction

two fungi (*Cryptococcus neoformans*, accession #L05428, and *Bullera unica*, accession #D78330), potential epibionts from Mollusca and Crustacea (*Mytilus galloprovincialis*, accession #L33451, and *Stenocypris major*, accession #Z22850), and a zooxanthella symbiont (*Symbiodinium* sp., accession #M88509). Octocoral sequences derived from frozen tissue in this lab (*Renilla reniformis* accession #AF052581, *Narella nuttingi* accession #AF052882, and *Anthomastus* sp. accession #AF052881) were verified as cnidarian through a BLAST search of GenBank and were also used in the primer design. Final DNA sequences were verified as cnidarian by a BLAST search of Genbank.

The specific primer pair combinations were selected to 1) amplify the largest region of the 18S gene possible for each specimen, and 2) include at least one octocoral-specific primer for one of the PCR reactions, reducing the probability of amplifying contaminating DNA. A diagram of the PCR primers used can be found in Fig. 1. If the initial PCR attempts were unsuccessful using primers A and B, a reamplification using internal primers was performed. If this was unsuccessful as well, I began anew with the initial 1:10 diluted DNA extract, and used PCR primers flanking a smaller genomic region. For example, an initial amplification would target the region flanked by primers A and 705R, and then the re-amplification would use primers A and 536R. The smallest fragment attempted for DNA sequence determination was approximately one third of the entire gene.

DNA Cloning and Sequencing Protocols

The final PCR product was cloned using the Original TA Cloning Kit (Invitrogen Corporation). The PCR product was ligated into the pCR 2.1 cloning vector, then transformed into a strain of INV α F' cells following the manufacturer's protocol. The plasmid DNA was isolated from individual clones using the Wizard Miniprep DNA Purification Kit (Promega Corporation) and subsequently used as a template for a cycle sequencing reaction, using the SequiTherm EXCEL Long-Read DNA Sequencing Kit-LC

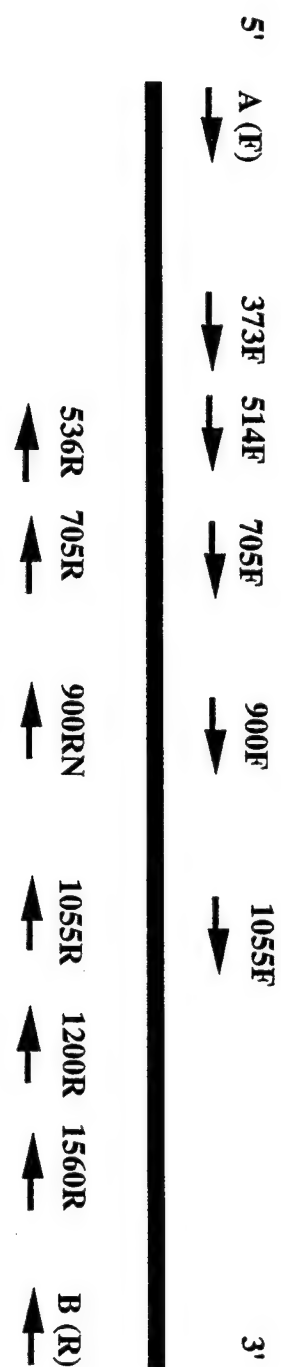


Fig. 1 Position and orientation of 18S rDNA primers used in PCR amplifications.

(Epicentre Technologies). Sequences were determined for both the forward and reverse strands of the gene. The reactions were run on a LI-COR 4000 DNA Sequencer, using the infrared-labeled primers shown in Table 2. The resulting images were interpreted using the BioImage gel reader program.

Negative Controls

I performed several controls throughout my work to detect any potential contamination. A negative extraction (no tissue added) was performed with the DNA extraction protocol. Negative controls (no DNA template added) were included with each primary and secondary PCR reaction. As one additional control, one frozen specimen and one ethanol-preserved specimen were chosen for re-extraction and re-determination of the DNA sequence to verify our ability to replicate the complete process. In each case, I found very good internal consistency. There were no sequence differences between the replicates for the frozen specimen (*Protophilum* sp.), and there was a 0.11% error rate, corresponding to 2 base changes over 1800 bp total for the ethanol-preserved specimen (*Umbellula* sp. USNM 54597) (Table 4).

Results

DNA extractions from tissues of ethanol-preserved museum specimens typically yielded small amounts of DNA that could rarely be detected on an agarose gel (Fig. 2, lanes 4 and 5). If DNA could be detected, it was of lower molecular weight than extractions of fresh tissue (Fig. 2, lane 3). My initial PCR amplification of the 1800-bp 18S rRNA gene from these extractions likewise did not yield visible product in the majority of museum specimens I examined. In some instances I was successful in amplifying smaller fragments (700-1200 bp) in a single PCR amplification. For the remainder of specimens for which a

Primer	Sequence
M13F*	5' CACGACGTTGTAACGAC 3'
M13R**	5' GAATAACAATTCACACAGG 3'
A (F)	5' ACCTGGTGATCCTGCC 3'
B (R)	5' CTTCTGCAGGTTCACTAC 3'
514F	5' TCTGGTGCCAGCASCCTGG 3'
536R	5' TGGWATTACCGCGGTGCTG 3'
1055F	5' GTGGTGGTGCATGGCCG 3'
1055R	5' AAGAACGGCCATGCACCCAC 3'

*F sequences prime in the 5' -> 3' direction

**R sequences prime in the 3' -> 5' direction

Table 2 Infrared-labeled primers used with the Licor sequencer. Primer numbers refer to position in prokaryotic small-subunit ribosomal DNA.

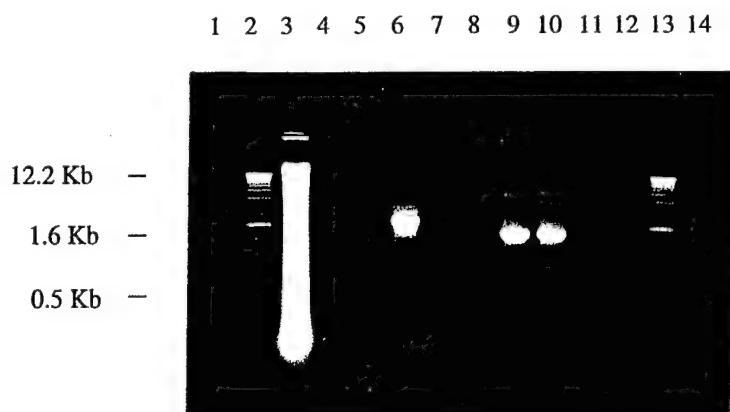


Fig. 2 Products of DNA extractions of museum specimens, initial PCR reactions, and secondary PCR reactions. Lanes are as follows: 1) blank; 2) 1-Kb ladder (Gibco BRL); 3) 5 μ l of *Palythoa variabilis* DNA extraction, tissue frozen; 4) 5 μ l of *Dendrobrachia paucispina* DNA extraction, tissue preserved in ethanol 8 years; 5) 5 μ l of *Nidalia occidentalis* DNA extraction, tissue preserved in ethanol 13 years; 6) Initial PCR reaction of *Palythoa variabilis* with primers A and B; 7) Initial PCR reaction of *D. paucispina* with primers A and B; 8) Initial PCR reaction of *N. occidentalis* with primers A and B; 9) Secondary PCR reaction of *D. paucispina* with primers A and 1200R; 10) Secondary PCR reaction of *N. occidentalis* with primers A and 1200R; 11) Secondary PCR reaction of negative control from primary PCR reaction, with primers A and 1200R; 12) Negative control from secondary PCR reaction with primers A and 1200R; 13) 1-Kb ladder (Gibco BRL); 14) blank.

single PCR reaction was unsuccessful, I performed a second PCR reaction using internal primers and the first PCR product as template (Fig. 2). The smallest fragment size I chose to amplify ranged from 500 to 700 bp.

The quality of DNA extracted from different museum specimens was evaluated based on the intensity of the DNA fragment as visualized on an agarose gel stained with ethidium bromide. This quality was highly variable, with no apparent correlation between the length of PCR product produced and the length of preservation time. I amplified 700-bp fragments from one specimen that had been preserved for 50 years, but was unable to amplify the same fragment from a specimen that had been preserved for nine years. Table 3 summarizes the results of DNA extractions and amplifications from the museum specimens. I did not perform PCR reamplifications on all samples, as I concentrated on those species I deemed phylogenetically important for our objectives. Archival specimens of those species for which fresh or recently preserved tissue became available were abandoned if PCR products were not readily produced. The variable success rate that I experienced in producing PCR products may have resulted from inconsistent handling of the specimens at the time of collection. This inconsistency may include preservation in formalin with subsequent transfer to alcohol. Such information is not always available for museum specimens.

I noted occasional nucleotide sequence variation within an individual specimen, both in overlapping regions of fragments produced in different PCR reactions and in pooled clones from the same PCR reaction. These base differences were found in most, but not in every one of the museum specimens (13 out of 15). The base differences I found consisted of simple substitutions, with only one instance of an insertion (four bp in *Dendrobrachia paucispina*). The resulting error was low (Table 4), usually less than 1%. This rate of error would apply to the remainder of the gene proportionally, not just to the

Table 3

Summary of results from all museum specimens. Specimens are from the National Museum of Natural History collections unless otherwise noted. Those specimens for which DNA sequences were generated are listed with the primer pairs used for the amplification reactions. Multiple primer pairs were used for those specimens that could not be amplified in one piece. If contaminant sequences were generated, the type of contaminant is identified. Specimens that yielded only contaminant sequences are noted with an asterisk. The specimens that did not generate sequences are also listed. PCR reamplifications were not attempted on all specimens as I concentrated on those species of greatest phylogenetic importance to my study. The most stringent attempt made for each specimen is noted.

- a: Harvard Museum of Comparative Zoology**
- b: Bermuda Aquarium, Natural History Museum and Zoo**
- c: Specimen was dried.**
- d: Specimen was abandoned when alternate specimens became available.**
- e: Sequence produced was a pseudogene or chimera.**

Species	Specimen #	Date Preserved	Cnidarian Sequences Generated	Initial PCR Amplifications	PCR Reamplifications Not Required (N/R)	Contaminant Found
<i>Tubipora musica</i>	USNM 79459	1947	yes	A/536R, 514F/1055R 1055F/B		algal
<i>Carica risel</i>	USNM 49691	1950	no ^d			
<i>Helopora coerulea</i>	not cataloged	1953	no*			fungal/cnidarian
<i>Itaiukella yanii</i>	USNM 51521	1953	no*			fungal/cnidarian
<i>Pachyclavaria violacea</i>	USNM 79630	1953	no	A/536R		
<i>Brilareum steebel</i>	USNM 50857	1954	no	A/B	900F/B	
<i>Clavularia koellikeri</i>	USNM 75090	1955	no	A/536R		
<i>Umbellula sp.</i>	USNM 87918	1961	no	A/B	900F/B	
<i>Keroelides koreni</i>	USNM 83605	1964	no	A/705R	A/536R	
<i>Eunicella albatrossi</i>	USNM 57212	1965	no	A/536R		
<i>Keratois sp.</i>	USNM 57283	1965	no ^d			
<i>Phidodilinda gardineri</i>	USNM 60217	1965	no	A/1200R	A/536R	
<i>Alcyonium palmatum</i>	USNM 57131	1967	no	A/536R		algal/fungal
<i>Belionella rubristella</i>	USNM 55382	1968	no*			fungal*
<i>Siphonogorgia sp.</i>	USNM 55582	1968	yes			
<i>Solenopodium sp.</i>	USNM 55664	1968	no ^d			
<i>Chrysogorgia spiculosa</i>	USNM 52854	1969	no*			fungal
<i>Thelagorgia stellata</i>	USNM 55443	1970	no*			fungal
<i>Flitsea paniculata</i>	USNM 93934	1971	yes	A/1200	A/536R	fungal
<i>Umbellula sp.</i>	USNM 54656	1971	yes	A/B	A/900R, 900F/B	
<i>Stephanogorgia wainwrightii</i>	USNM 54056	1972	no ^d			
<i>Umbellula sp.*</i>	N/A	1972	yes	A/B	900F/B	
<i>Belionella indica</i>	USNM 58498	1973	no ^d			
<i>Meilithaea ochracea</i>	USNM 58590	1973	no	A/536R		
<i>Siphonogorgia (Chilonephthya) sp.</i>	USNM 58584	1973	yes	A/900R, 705F/1200R 900F/B	A/705R, 705F/1055R 900F/B	
<i>Siphonogorgia godeffroyi</i>	USNM 58581	1973	no	A/705R	A/536R	
<i>Umbellula thomsonii</i>	USNM 58994	1973	no	A/B	900F/B	
<i>Talaria tauhou</i>	USNM 54271	1974	yes	A/536R, 514F/1055R 1055F/B	N/R	
<i>Umbellula sp.</i>	USNM 54597	1974	yes	A/B	N/R	fungal
<i>Acanthogorgia armata</i>	USNM 55837	1975	no*			fungal/protozoan
<i>Australis sarmentosa</i>	USNM 78370	1975	no*			
<i>Brilareum asbeslinum</i>	USNM 59051	1975	no	A/536R		
<i>Renilla reniformis</i>	USNM 57404	1975	no	A/536R		
<i>Stereonephthya unicolor</i>	USNM 81528	1975	no*			algal/yeast
<i>Piumigorgia astroleptes</i>	USNM 76298	1976	yes			e
<i>Nitulia occidentalis</i> ^e	19-011-01	1977	yes	A/B, 900F/B	A/1200R, 1055F/B	

Species	Specimen #	Date Preserved	Cnidarian Sequences Generated	Initial PCR Amplifications	PCR Reamplifications Required	Contaminant Found
<i>Xenia</i> sp.	USNM 79620	1978	no	A/536R		
<i>Leptogorgia virgulata</i>	USNM 88494	1981	no*			fungal/plant
<i>Nicella quadalupensis</i>	USNM 73722	1981	no	A/B	900F/B	
<i>Telesto fruticulosa</i>	USNM 61122	1981	yes	A/B	A/900R, 900F/B	
<i>Titanidium frauentzilli</i>	USNM 88222	1981	no	A/1200R	A/536R	
<i>Carloa nisei</i>	USNM 86021	1982	no	A/B	900F/B	
<i>Coelogorgia palmosa</i>	USNM 75637	1983	yes	A/705R	A/536R	fungal/bivalve
<i>Nidella occidentalis</i>	USNM 74730	1983	no ^d			
<i>Plexaurella nutans</i>	USNM 84101	1983	no ^d			
<i>Telesto fruticulosa</i>	USNM 88959	1983	no*			bivalve
<i>Gorgonia ventalina</i>	USNM 88767	1984	yes			symbiont/fungal*
<i>Junceella racemosa</i>	USNM 92412	1984	yes	A/B	N/R	bivalve/fungal/cnidarian
<i>Subergorgia suberosa</i>	USNM 75340	1984	no*			bivalve
unidentified sp.	unidentified	1984	no*			
<i>Clavularia modesta</i>	USNM 79765	1985	no	A/705R		
<i>Eumicea lacinata</i>	USNM 73614	1985	yes	A/B	A/1200R, 373F/B	
<i>Isis hippuris</i>	USNM 80861	1985	no	A/536R		
<i>Mellita</i> sp.*	N/A	1985	yes	A/705R, 705F/1200R A/B	705F/1055R, 900F/B	bivalve
<i>Orstomis crosnieri</i>	USNM 84774	1985	yes	A/B, 373F/B	A/1200R	
<i>Metalloporgia</i> sp.	USNM 89377	1986	no*			fungal/algal
<i>Helopora coerules</i>	USNM 79529	1987	yes	373F/B, A/705R, 900F/B	514F/1055R, A/536R, 1055F/B	
<i>Pennatula grandis</i>	USNM 79776	1987	no ^d			
<i>Solenopodium excavatum</i>	USNM 88852	1987	no*			fungal/bacterial
<i>Nicella obesa</i>	USNM 84332	1988	no	A/536R		
<i>Dendrobrachia paucispina</i>	USNM 97768	1989	yes	A/B	A/1200R, 373F/B	fungal
<i>Junceella gemmacea</i>	USNM 87607	1989	no	A/B	900F/B	
<i>Epiphaxum breve</i> *	USNM 91942	1990	yes	A/705R, 900F/B	A/536R, 1055F/B	bacterial/algal
<i>Piumigorgia hydroides</i> *	N/A	1992	yes	373F/1560R, 900F/B	514F/1055R, 1055F/B	
<i>Calcigorgia spiculifera</i>	not cataloged	1994	yes	A/B	N/R	

Table 4	<p>Error rates found in sequences of museum specimens, as seen in the pooling of multiple clones from the same PCR reaction, and overlapping fragments produced from different PCR reactions. (Note: the forward and reverse strands of a single clone and a single fragment showed no sequence differences.) The species listed here are the only museum specimens for which there were overlapping regions or pooled clones.</p> <p>a: Error rate within a single PCR reaction as detected from pooling multiple clones.</p> <p>b: Error rate between PCR reactions as detected in fragments shared by overlapping clones.</p>
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a)

Specimen	ID Number	Date Preserved	Size of pooled clones (bp)	Number of Transitions	Number of Transversions	Resulting Error (%)
<i>Umbellula</i> sp.	USNM 54656	1971	505, 422	3	2	0.54
<i>Siphonogorgia</i> (<i>Chironephthya</i>) sp	USNM 58584	1973	620, 660	6	2	0.63
<i>Nidalia occidentalis</i>	09-011-01	1977	531, 1430	7	0	0.36
<i>Telesto fruticulosa</i>	USNM 61122	1981	505, 265	1	0	0.13
<i>Juncella racemosa</i>	USNM 92412	1984	505, 545	8	0	0.76
<i>Melitella</i> sp.	MCZ	1985	694, 628	9	2	0.83
<i>Heliopora coerulea</i>	USNM 79529	1987	603, 533, 758	17	6	1.21
<i>Dendrobrachia paucispina</i>	USNM 97768	1989	865	1	1	0.23
<i>Epiphaxum breve</i>	USNM 91942	1990	530	1	0	0.19
<i>Plumigorgia hydroides</i>	MCZ	1992	710, 525	4	0	0.32
<i>Calcigorgia spiculifera</i>	USNM not cat.	1994	910	0	0	0.00

b)

Specimen	ID Number	Date Preserved	Length of Overlap (bp)	Number of Transitions	Number of Transversions	% Error in Overlap
<i>Siphonogorgia</i> sp.	USNM 55582	1968	459	20	7	5.88
<i>Siphonogorgia</i> (<i>Chironephthya</i>) sp	USNM 58584	1973	450	2	0	0.44
<i>Umbellula</i> sp.	USNM 54597	1974	1800*	0	2	0.11
<i>Nidalia occidentalis</i>	09-011-01	1977	175	0	0	0.00
<i>Juncella racemosa</i>	USNM 92412	1984	507	0	2	0.39
<i>Orstomis crosnieri</i>	USNM 84774	1985	985	1	1	0.20
<i>Eunicea lacinata</i>	USNM 73614	1985	1015	1	0	0.10
<i>Melitella</i> sp.	MCZ	1985	445	0	2	0.45
<i>Dendrobrachia paucispina</i>	USNM 97768	1989	1069	0	1	0.01
<i>Protopilum</i> sp.	USNM	1993	1800*	0	0	0.00
<i>Calcigorgia spiculifera</i>	USNM not cat.	1994	448	2	1	0.67

* Comparing results of replicate extractions of same initial specimen

regions containing these overlaps. The one sequence that showed a high rate of PCR error (5.88% in *Siphonogorgia* sp.) was not used in subsequent phylogenetic analyses.

Several initial attempts at obtaining DNA sequences from museum specimens using universal eukaryotic primers yielded non-cnidarian sequences as indicated through BLAST searches. The contaminant sequences generated were of the algal symbiont *Symbiodinium* sp. and other algal species, various fungi, and occasionally bivalve mollusks and crustaceans. These species were potential symbionts or epibionts of the coral specimens we were analyzing. The use of taxon-specific PCR primers eliminated the amplification of contaminant DNA completely.

Discussion

The protocol outlined here has been successful for generating sequence information from museum specimens preserved up to fifty years. Most genomic DNA extractions from ethanol-preserved specimens were partially degraded, and two rounds of PCR amplifications were required to generate sufficient PCR product for further analyses. The probability of obtaining DNA sequences from contaminant DNA rather than target DNA was significant without the use of taxon-specific primers. The problem of generating non-Cnidarian sequences was eliminated when taxon-specific primers were used in at least one of the two PCR reactions.

A major concern I had with the use of archival specimens was the occasional differences found in overlapping regions of sequence within the same individual. *Taq* DNA polymerase has an inherent rate of error associated with it, which may account for some, if not all, of the replication error observed. Two estimations of the rate of error connected with the use of *Taq* polymerase are 8.0×10^{-6} (errors per base per PCR cycle) (Cline et al. 1996) and 2.1×10^{-4} (Keohavong and Thilly 1989). These error rates applied to the 18S rRNA gene of anthozoans, translate to 0.028%-0.735% error in 35 replication

cycles over the entire gene. For those specimens that were reamplified, the estimated error rate would be 0.056%-1.47% over 70 replication cycles. Only one of the museum specimens exhibited error rates greater than these (5.88% for *Siphonogorgia* sp.). The increased error rate for this specimens may have been due to the presence of damaged DNA. Although the activity of DNA polymerases is slowed by the presence of damaged DNA, particularly at baseless sites, minor lesions such as deaminated bases can produce replication errors without slowing the replication process greatly (Paabo 1989). Therefore, DNA with minor damage will be amplified at the same rate as undamaged DNA. Direct-sequencing of total PCR products will reduce the influence of either type of replication error on the final DNA sequence, since any errors introduced after all but the first rounds of PCR will be evident as ambiguities (Hillis et al. 1996).

PCR amplifications of archival, preserved specimens may not produce sufficient PCR product for direct-sequencing techniques, and cloning of PCR products may be necessary. Since each clone consists of a single PCR product which may contain replication errors, the potential for generating incorrect sequences is heightened (Palumbi, 1996). This is particularly true for these archival specimens since two PCR amplifications were necessary for obtaining DNA sequences from the majority of these samples, thereby increasing the risk of polymerase-introduced errors. This risk makes it particularly important to pool several clones before performing the DNA sequencing reactions; as with determination of sequences directly from PCR products, errors will be evident as ambiguities in the sequence (Medlin et al. 1988). The overall rate of PCR error can then be determined for a given specimen. Depending on the taxonomic scale of phylogenetic analysis being performed, the amount of error found may or may not be significant in the overall analyses. Any amount of error is of concern, however, and one should be aware of the increased chance of such errors when working with preserved specimens. This is

especially true when analyzing gene flow, as the amount of error may be greater than the genetic signal present.

Protocols for extracting DNA from alcohol-preserved samples are readily available in the literature (Smith et al. 1987; Palumbi et al. 1991), however, very few studies have been published to date using archival ethanol-fixed specimens. The Smith et al. (1987) protocol did not use samples that had been preserved longer than six years. One notable study that used older ethanol-preserved specimens, however, was Persing et al.'s (1990) discovery of the presence of spirochete DNA responsible for Lyme disease in ticks that had been preserved for nearly 50 years. These ticks had been collected 30 years prior to the first documentation of the disease in the United States. Persing et al. (1990) were amplifying very small regions of the mitochondrial genome, however, ranging from 77-200 bp. Fragments this small are sufficient for the detection of the Lyme Disease spirochete genome, but are too small for reliable large-scale phylogenetic studies. The protocol described here allows the generation of larger genomic fragments, which will be useful in studies involving phylogenetics and gene flow, among others.

The phylogenetic analysis of octocoral species is only one of a vast number of studies that can benefit from molecular techniques as applied to archival specimens. I submit here a method that has been successful in generating relatively large PCR fragments of DNA from archival, long-preserved octocoral specimens. There are some general concerns when working with museum specimens, however. My success rate from sample to sample was highly variable, and I did see a small but measurable incidence of PCR-induced sequence error. One is also more likely to encounter the effects of background contamination in the samples when working with older specimens. Despite such difficulties, the use of protocols such as the one outlined above can facilitate the generation of valuable genetic information from preserved museum specimens.

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Chapter 3

Phylogenetic Relationships within the Class Anthozoa (Phylum Cnidaria) Based on Nuclear 18S rDNA Sequence Information

Abstract

Taxonomic relationships within the corals and anemones (Phylum Cnidaria: Class Anthozoa) are based upon few morphological characters: colony morphology and the structure of the tentacles, gastric mesenteries, nematocysts, and skeletal axis. The significance of any given character is debatable, and there is little fossil record available for deriving evolutionary relationships. In this study I use complete sequences of 18S ribosomal DNA to examine subclass-level and ordinal-level organization within the Anthozoa. I investigate whether the traditional two-subclass system (Octocorallia, Hexacorallia) or the current three-subclass system (Octocorallia, Hexacorallia, Ceriantipatharia) is better supported by sequence information. I also examine the phylogenetic affinities of the anemone-like species *Dactylanthus antarcticus* (Order Ptychodactiaria) and the putative antipatharian *Dendrobrachia paucispina*. Thirty-eight species were chosen to maximize the representation of morphological diversity within the Anthozoa. Maximum likelihood techniques were employed in the analyses of these data, using relevant models of evolution for the 18S rRNA gene. I conclude that placing the orders Antipatharia and Ceriantharia into the Subclass Ceriantipatharia does not reflect the evolutionary history of these orders. The Order Antipatharia is closely related to the Order Zoanthidea within the Hexacorallia and the Order Ceriantharia appears to branch early within the Anthozoa, but the affinities of the Ceriantharia cannot be reliably established from these data. My data generally support the two-subclass system, although the Ceriantharia may constitute a third subclass on their own. The Order Corallimorpharia is likely polyphyletic, and its species are closely related to the Order Scleractinia. *Dactylanthus*, also within the Hexacorallia, is allied with the anemones in the Order Actiniaria, and their current ordinal-level designation does not appear to be justified. The genus *Dendrobrachia*, originally classified within the Order Antipatharia, is closer

phylogenetically to the Subclass Octocorallia. The 18S rRNA gene may be insufficient for establishing concrete phylogenetic hypotheses concerning the specific relationships of the Corallimorpharia and the Ceriantharia, and the branching sequence for the orders within the Hexacorallia. The 18S rRNA gene has sufficient phylogenetic signal, however, to distinguish among the major groupings within the Class Anthozoa, and I can use this information to suggest relationships for several enigmatic taxa.

Introduction

The development of taxonomic systems through traditional, morphological methods can be problematic when the species involved have few distinguishing characters. This is true for species within the Class Anthozoa (Phylum Cnidaria). The Class Anthozoa, containing the stony corals, soft corals, anemones and other anemone-like species, retain their polyp morphology throughout their life history, and lack the medusa stage found commonly in the other classes of the phylum. The primary characters that have been used to derive evolutionary relationships within the Anthozoa include colony morphology and life history, tentacle shape and number, the number and arrangement of divisions within the gastrovascular cavity (termed mesenteries or septa), nematocyst structure, and skeletal structure. The various taxonomic arrangements have arisen from differing interpretations of the significance of these characters (e.g. Wells & Hill 1956a, Hadzi 1963, discussed below). As many Anthozoa lack any type of skeletal structure, there is little fossil record to indicate evolutionary relationships among the major groups of anthozoans.

The validity of the subclass divisions within the Anthozoa has been a subject for debate by taxonomists of both past and present times. The historical subclass divisions within the Class Anthozoa indicate two major groupings, the Subclass Octocorallia and the Subclass Hexacorallia (e.g. Minchin et al. 1900, Pratt 1935, Hyman 1956, Hadzi 1963). The Subclass Octocorallia is composed of the soft corals, gorgonians, blue corals, and sea

pens. Octocorals are distinguished by their possession of eight pinnately branched tentacles and eight complete mesenteries. Their skeletons may contain separate or fused calcium carbonate spicules, and axes of calcium carbonate or chitin or both, and they may be solitary or colonial (Bayer 1956, Wells & Hill 1956a). The Subclass Hexacorallia, in its early definition, contained the remaining anthozoan species: the Actiniaria (anemones), Corallimorpharia, Zoanthidea, Scleractinia (stony corals), Antipatharia (black corals), and Ceriantharia (cerianthid anemones). The subclass was defined by the following characteristics: tentacles simple or divided, but never branched; paired mesenteries, usually in multiples of six; skeleton, if present, without free spicules in the mesoglea. Hexacorals include both solitary or colonial forms.

A third subclass division was proposed as early as 1897, although it wasn't widely accepted until the 1950's or 1960's. The orders Antipatharia and Ceriantharia were removed from the Subclass Hexacorallia and placed in a new subclass, the Ceriantipatharia. The establishment of the Subclass Ceriantipatharia was based primarily on two shared characters between the orders Ceriantharia and Antipatharia: 1) the resemblance of the cerianthid larva to the antipatharian adult polyp, and 2) weak and indefinite musculature associated with the mesenteries (van Beneden 1897). Aside from the above characters, these two orders are considered highly divergent from each other (Wells & Hill 1956b). The Ceriantharia have a unique morphology which distinguishes them from the other Anthozoa. They have two rings of tentacles, and numerous, mostly complete, unpaired mesenteries (Hyman 1956). They possess a unique form of spirocysts (ptychocysts) not present in any other order within the class (Fautin & Mariscal 1991, Rifkin 1991, Goldberg & Taylor 1996). The Ceriantharia have been designated as most primitive within the Hexacorallia, most deeply diverging (Hyman 1940).

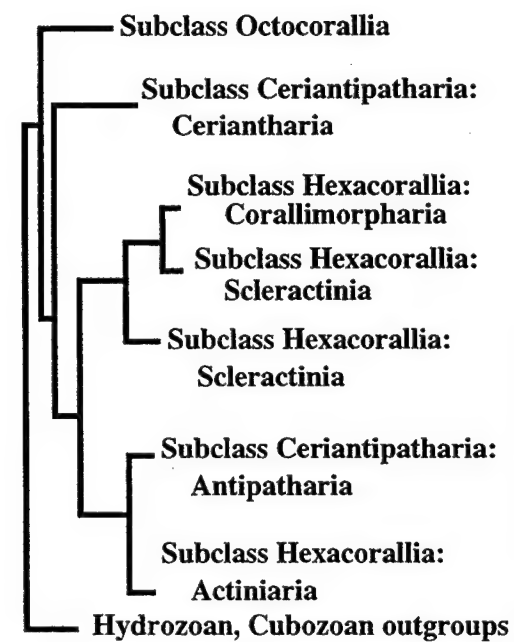
The Antipatharia, placed with the Ceriantharia in the Subclass Ceriantipatharia, have been considered to be ancestral with the cerianthids (Wells & Hill 1956a) or highly derived

(recently divergent) hexacorals (Brook 1889, Hickson 1906, Hadzi 1963). Morphological characters suggest affinities among the Antipatharia, Zoanthidea and Actiniaria. All three orders share a type of nematocyst, a microbasic b-mastigophore, which is rare or absent in the other orders of the Anthozoa (Picken & Skaer 1966). Antipatharians, zoanthids, and the Subtribe Endomyaria of the Actiniaria share a common sperm morphology, which is not found in the other anthozoans. The Antipatharia and the Zoanthidea also share similarities in nematocyst structure and skeletal composition (Schmidt 1974), and are thought by some to be highly specialized hexacorals (Hadzi 1963).

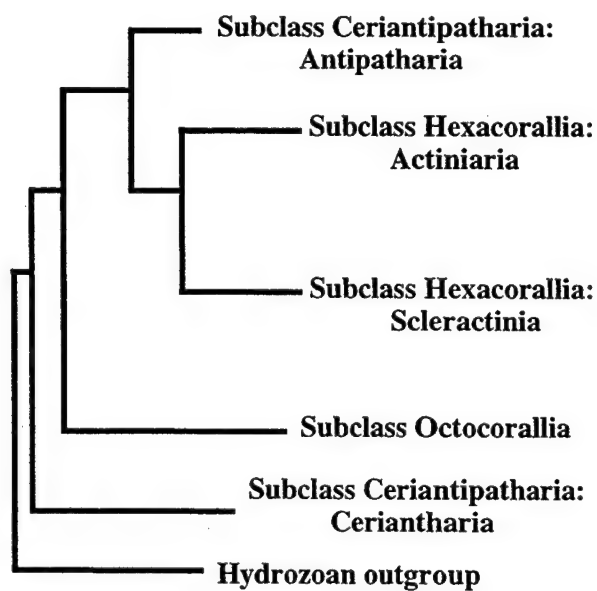
Molecular studies using mitochondrial 16S rDNA (France et al. 1996) and 18S rDNA (Song & Won 1997) have shown that the orders Antipatharia and Ceriantharia are genetically divergent from one another, and are not sister orders. The results of these studies disagreed on the positions of the Ceriantharia and Antipatharia relative to the other Anthozoa, however (Fig. 1). A study using 28S rDNA (Chen et al. 1995) and another study combining mt 16S rDNA and 18S rDNA (Bridge et al. 1995), included a single cerianthid as the representative of the Subclass Ceriantipatharia, so would have been unable to detect any potential divergence between the Ceriantharia and the Antipatharia. None of these studies included representatives of the Zoanthidea. Resolution of the phylogenetic affinities and ancestry of the orders Antipatharia and Ceriantharia will further our understanding of the early evolution of the Anthozoa, and may help determine which morphological characters are phylogenetically informative.

The phylogenetic relationships among the orders Corallimorpharia, Scleractinia, and Actiniaria are equivocal based on morphological characters. The Corallimorpharia are morphologically intermediate between the actinarians and the scleractinians, although their mesentery structure and nematocysts are closer to the scleractinians (Fautin & Lowenstein 1992). At one time, the Corallimorpharia were placed within the same order as scleractinians (Wells & Hill 1956c). In the late 1800's the corallimorpharian species

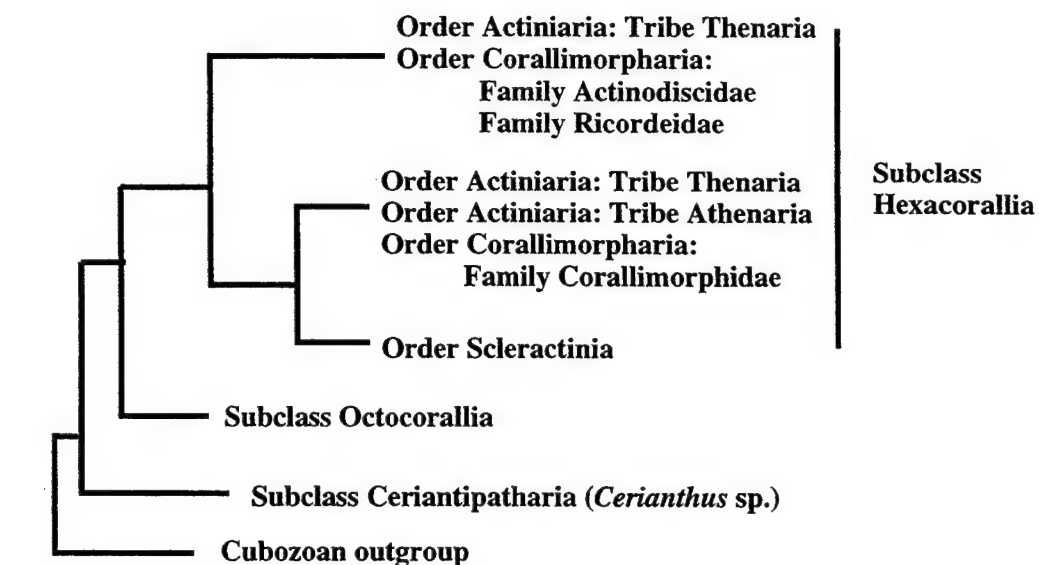
Fig. 1 **Phylogenetic trees from previous molecular studies of the Anthozoa.**
A) **From France et al. (1996), based on mitochondrial 16S rDNA**
B) **From Song and Won (1997), based on nuclear 18S rDNA**
C) **From Chen et al. (1995), based on nuclear 28S rDNA**



A



B



C

Corynactis was suggested to be an immature scleractinian (Jourdan 1880). The Corallimorpharia have also been considered to be part of the Actiniaria historically (Carlgren 1949). Stephenson (1921) removed the corallimorpharian species from the Actiniaria and designated them as Madreporaria (containing the Corallimorpharia and Scleractinia). Stephenson perceived the Corallimorpharia as Madreporaria that never developed skeletons. Carlgren (1949) established the Corallimorpharia as a separate order. The current scenario gives the three groups equal distinction at the ordinal level within the Subclass Hexacorallia, implying that the presence or absence of a skeleton has greater relevance in identifying phylogenetic affiliations than internal morphology.

Molecular studies have reached different conclusions, however, concerning the relationships among the Actiniaria, Scleractinia, and Corallimorpharia. Partial 28S rDNA sequences (Chen et al. 1995) suggested monophyly of the Scleractinia, and polyphyly of the Corallimorpharia and the Actiniaria (Fig. 1C). Protein radioimmunoassay analyses (Fautin & Lowenstein 1992) indicated that the Corallimorpharia are not distinct from the Scleractinia. Fautin and Lowenstein (1992) hypothesized that the Corallimorpharia may have had multiple origins within the Scleractinia. Mitochondrial 16S rDNA also suggested that the Corallimorpharia branch within the Scleractinia (France et al. 1996).

My study investigated whether the Corallimorpharia were allied with the Scleractinia, the Actiniaria, or neither. The determination of the phylogenetic affinities of the Corallimorpharia should help resolve whether the presence of a skeleton or the internal morphology is more indicative of evolutionary history.

The validity of the current ordinal distinction for the Ptychodactiaria, a group of anemone-like species, is another systematics issue that remains unresolved. The ptychodactarian species were originally classified as a family within the Actiniaria, included in Protantheae with the Gonactiniidae based on similarities in nematocysts and primitive musculature (Stephenson 1921). The Ptychodactiaria differed from the

Actiniaria, however, in their gonadal arrangement and mesenterial structures (Stephenson 1921, Wells & Hill 1956c). Stephenson (1921, 1922) removed the Ptychodactidae from the Protantheae and created a third tribe, the Ptychodactae. Carlgren (1949) gave the Ptychodactiaria ordinal ranking, arguing that these characteristics were primitive rather than degenerate. The present work seeks to gain insight not only to the amount of support for their ordinal standing, but also the degree to which these species are ancestral or derived.

The genus *Dendrobrachia* exhibits an interesting combination of morphological characters that resemble both the Antipatharia and the Octocorallia. The *Dendrobrachia* specimen collected in the Challenger Expedition (1872-1876) was in relatively poor condition, but was assigned to the Order Antipatharia based on its chitinous, spiny axis and its lack of sclerites (Brook 1889). *Dendrobrachia* has always been recognized, however, as an 'aberrant' antipatharian (e.g. van Beneden 1897). Additional specimens of *Dendrobrachia* have been shown to possess several characteristically octocorallian features: eight pinnately branched, retractile tentacles, and a solid axial core (Opresko & Bayer 1991). Opresko (1991) suggested that Dendrobrachiidae be established as a family within the Octocorallia, with affinities to the gorgonians. This study will determine whether *Dendrobrachia* is more closely related to the Order Antipatharia (Subclass Ceriantipatharia or Hexacorallia) or the Subclass Octocorallia, and again whether the skeletal morphology is more important evolutionarily than other morphological characters.

For the molecular phylogenetic analyses in the present study I used Maximum Likelihood (ML) techniques, which have only recently become tractable for large data sets (Huelsenbeck & Crandall 1997, Huelsenbeck & Rannala 1997). These methods are appropriate for the analysis of sequence data, as the evolutionary algorithm can be tailored specifically to the gene analyzed (Swofford et al. 1996). As a result, the number of incorrect assumptions about the evolution of the gene can be minimized in the phylogenetic analyses. Without such specific ML methods, the error introduced into phylogenetic

analyses from incorrect assumptions can overwhelm the phylogenetic signal present in the data. This can be a serious problem when working with large numbers of taxa or basepairs (DeSalle et al. 1994, Rzhetsky & Nei 1995). Methods incorporating too many incorrect assumptions will tend to converge on an incorrect tree, given increasing amounts of sequence information (Huelsenbeck & Crandall 1997). For example, using a model employing equal substitution rates within a gene where it is not appropriate can bring the probability of finding the correct tree to zero with sequences greater than 2,000 bp (Sullivan & Swofford 1997). In addition, maximum likelihood analyses produce a statistic (the likelihood score) that can be useful for comparing specific evolutionary hypotheses (i.e. phylogenetic trees). This statistic will indicate if a given hypothesis is significantly better or worse than an alternative (Hillis 1995, Huelsenbeck & Rannala 1997).

The previous phylogenetic studies of the Anthozoa had limitations for addressing the scope of questions I ask here. All of the studies had limited taxon sampling within the Hexacorallia (Bridge et al. 1995, Chen et al. 1995, France et al. 1996, Song & Won 1997) or Ceriantipatharia (Bridge et al. 1995, Chen et al. 1995), and included only partial gene sequences. These studies used relatively basic phylogenetic analyses (parsimony and distance) employing evolutionary models that are not highly accurate with respect to the data set. The goal of my study was to build a more complete phylogeny of the Class Anthozoa than currently exists, containing representatives from across the morphological breadth present within the class, using complete 18S rDNA sequences. This study addresses a number of questions regarding anthozoan phylogeny: 1) are the Ceriantharia and the Antipatharia phylogenetically allied within the Subclass Ceriantipatharia, separate from the Subclass Hexacorallia, 2) are the Corallimorpharia affiliated with the Actiniaria, the Scleractinia, or other members of the Hexacorallia, 3) are the Ptychodactiaria phylogenetically distinct from the Actiniaria, 4) is *Dendrobrachia* affiliated with the Antipatharia (Subclass Hexacorallia) or the Subclass Octocorallia, and 5) which

morphological characters are correlated most closely with phylogenetic divisions within the Anthozoa?

I chose the nuclear 18S rRNA gene for this study because it has been shown repeatedly to be useful in addressing questions on a variety of evolutionary scales, ranging from differentiation of kingdoms (e.g. Wainright et al. 1993) to elucidation of relationships within a given order (e.g. Kelly-Borges et al. 1991, Hay et al. 1995) or even within a single family (e.g. Kuznedelov & Timoshkin 1993, Fitch et al. 1995). I address some of the conflicting results from previous molecular studies using maximum likelihood analyses, which can incorporate relevant evolutionary assumptions for the 18S rRNA gene in anthozoans, and suggest hypotheses for issues where no molecular information has previously existed.

Methods

Specimens

This study included over 1600 basepairs (bp) of sequence information from the nuclear 18S rRNA gene of 40 anthozoan species plus 7 outgroup species, representing all of the extant orders of the Anthozoa. Fifteen of the sequences used were taken from Genbank, and the remaining 32 were determined for this study. The species came from a variety of sources (Table 1). Several octocoral specimens and the specimen of *Dendrobrachia paucispina* were acquired from Dr. Frederick Bayer of the National Museum of Natural History and Ardis Johnston of the Harvard Museum for Comparative Zoology. These specimens had been stored in ethanol for periods ranging from two to 50 years. No information was available as to whether the samples were fixed originally in ethanol or in formalin. Fresh specimens of octocorals, zoanthids, antipatharians, and actiniarians were collected by submersible from Hawaiian seamounts (using the Pisces V submersible, operated by the Hawaiian Undersea Research Laboratory at the University of Hawaii) and

Table 1 **Specimens used in phylogenetic analyses. Species of Placozoa, Porifera, Ctenophora, Hydrozoa and Cubozoa were used as outgroups. Specimen sources are as follows: Bishop Seamount, 18.8°N 159.1°W; CG, courtesy of Constance Gramlich, UCSD; EG, courtesy of Erica Goldman, University of Washington; Fieberling Guyot, 32.1°N 127.8°W; LP, courtesy of Dr. Lloyd Peck of the British Antarctic Program and Dr. Daphne Fautin of the Division of Invertebrate Zoology at Kansas University Natural History Museum; NMNH, provided by Dr. Frederick Bayer, National Museum of Natural History, Smithsonian; Pensacola Seamount, 18.3°N 157.3°W; SR, courtesy of Dr. Sandra Romano, University of Guam; WG, courtesy of Dr. Walter Goldberg, Florida International University.**

Species	Source	ID #	Genbank Accession #
Phylum Porifera			
Class Calcarea			
<i>Scypha ciliata</i>	Genbank		D15066
Class Demospongiae			
<i>Tetilla japonica</i>	Genbank		D15067
Phylum Ctenophora			
<i>Mnemiopsis leidyi</i>	Genbank		L10826
<i>Beroe cucumis</i>	Genbank		D15068
Phylum Placozoa			
<i>Trichoplax</i> sp.	Genbank		Z22783
Phylum Cnidaria			
Class Hydrozoa			
<i>Selaginopsis cornigera</i>	Genbank		Z92899
Class Cubozoa			
<i>Tripedalia cystophora</i>	Genbank		L10829
Class Anthozoa			
Subclass Octocorallia			
Order Pennatulacea			
Suborder Sessiliflorae			
Fam. Protoptilidae	Bishop Seamount, HI, 1440 m	BI101-3	AF052911
<i>Protopitium</i> sp.			
Fam. Renillidae	Mission Bay, CA, 8 m ^{CG}		AF052581
<i>Renilla reniformis</i>			
Fam. Umbellulidae			
<i>Umbellula</i> sp.	Bahamas, 1447 m ^{NMNH}	54597	AF052904
Suborder Subselliflorae			
Fam. Virgulariidae			
<i>Acanthoptilum</i> sp.	Mission Bay, CA, 8 m ^{CG}		AF052910

Order Alcyonacea			
Suborder Protoalcyonaria			
Fam. Taiaroidae			
<i>Taiaira tauhou</i>	Otago Peninsula, NZ, 420-320 m ^{NMNH}	54271	AF052908
Suborder Stolonifera			
Fam. Tubiporidae			
<i>Tubipora musica</i>	Bikini Atoll, Marshall Islands ^{NMNH}	79459	AF052909
Suborder Alcyoniina			
Fam. Alcyonidae			
<i>Bellonella rigida</i>	Genbank		Z49195
Suborder Scleraxonia			
Fam. Briareidae			
<i>Briareum asbestinum</i>	San Blas Islands, Panama ^{TG}		AF052912
Suborder Holaxonia			
Fam. Acanthogorgiidae			
<i>Acanthogorgia</i> sp.	Bishop Seamount, HI, 1285 m	B11104-3	AF052907
Fam. Dendrobrachiidae			
<i>Dendrobrachia paucispina</i>	Great Australian Bight, 884-859 m ^{NMNH}	87768	AF052903
Fam. Isididae			
<i>Lepidisis</i> sp.	Pensacola Seamount, HI, 1425 m	PN1104-1	AF052906
Fam. Primnoidae			
<i>Narella bowersi</i>	Pensacola Seamount, HI, 1350 m	PN1105-3	AF052905
Subclass Hexacorallia			
Order Actinaria			
Suborder Nynantheae			
Tribe Thenaria			
Subtribe Endomyaria			
Fam. Actiniidae			
<i>Anemonia sulcata</i>	Genbank		X53498
<i>Anthopleura kurogane</i>	Genbank		Z21671
Subtribe Mesomyaria			
Fam. Actinostolidae			
<i>Stomphia</i> sp.	Friday Harbor, WA, subtidal ^{EG}		AF052888

Order Corallimorpharia		
Fam. Discosomatidae		
<i>Discosoma</i> sp.	Bermuda, subtidal	AF052894
Fam. Corallimorphidae		
<i>Corynactis californica</i>	aquarium specimen, University of New Hampshire	AF052895
Subclass Ceriantipatharia		
Order Ceriantaria		
Fam. Cerianthidae		
<i>Cerianthus borealis</i>	Nubble Lighthouse, ME, subtidal	AF052897
<i>Ceriantheopsis americanus</i>	Fort Pierce Inlet, FL, 0.5 m	AF052898
Order Antipatharia		
Fam. Antipathidae		
<i>Stichopathes spieessi</i>	Fieberling Guyot, 490 m	AD2296-14
<i>Antipathes fiordensis</i>	Bradshaw Sound, Fiordland NZ	AF052899
<i>Antipathes lata</i>	Genbank	AF052900
<i>Bathypathes</i> sp.	Penguin Bank, HI, 418 m	Z92908
<i>Cirripathes lutkeni</i>	Hollywood, FL, 25 m	AF052901
	WG	AF052902

Fieberling Guyot (using the Alvin submersible, operated by the Woods Hole Oceanographic Institution). The specimens collected from these dives were either frozen in liquid nitrogen or in a -20°C freezer. Other actinarian and corallimorpharian specimens were collected in U.S. and Bermuda by snorkeling or SCUBA. Two antipatharian specimens were donated by Dr. Walter Goldberg of the Florida International University. The ptychodactarian specimen was donated by Dr. Lloyd Peck of the British Antarctic Program and Dr. Daphne Fautin of the Division of Invertebrate Zoology at Kansas University Natural History Museum. DNA extractions of scleractinians and several octocorals were donated by Dr. Sandra Romano of the University of Guam Marine Station and Dr. Tamar Goulet of the State University of New York, Buffalo, respectively.

DNA Extraction Protocol

The DNA extraction protocols I used was similar to those described by Coffroth et al. (1992) and Winnepeninckx et al. (1993). Five to ten polyps of fresh or frozen tissues were minced with a razor blade and placed in a 1.5-ml eppendorf tube with 600 µl of 2X cetyltrimethylammonium bromide (CTAB) buffer (1.4M NaCl, 0.02M EDTA, 0.1M Tris-HCl (pH 8.0), 2% CTAB (Sigma Chemical Co.), and 0.2% beta-mercaptoethanol). This buffer is particularly effective at removing polysaccharides that are abundant in coral tissues, and which can interfere with DNA extraction. A plastic dounce was employed to shear the tissue further, and an additional 300 µl of 2X CTAB was added. The samples were placed at 55°C and digested with 5 µl of proteinase K (20 mg/ml) for approximately two hours. The tissues were extracted once with an equal volume of 24:1 chloroform:isoamyl alcohol, and precipitated in two volumes of cold 95% ethanol at -20°C overnight. The tubes were centrifuged at 10,000xg for 30 minutes, and the ethanol was removed. The pellets were washed with 500 µl cold 70% ethanol, and the tubes were centrifuged at 7,000xg for 15 minutes. The ethanol was removed, and the pellets dried at

room temperature. The pellets were resuspended in 50 µl of TE buffer (10 mM Tris-HCl--pH 8.0, 1 mM EDTA--pH 8.0) and placed at 4°C for three to four hours before visualization on an agarose gel.

The ethanol-preserved specimens were extracted with a slightly different protocol. Five to ten polyps of the archival specimens were placed on ice in two to ten ml 2X CTAB buffer for two to 24 hours, with the buffer replaced several times during this period. The buffer was removed, and the tissue minced finely with a razor blade as above. The tissues were incubated with proteinase K at 55°C for 24 hours, with periodic agitation. Another 5 µl of proteinase K was added, and the tissues continued to digest for an additional eight to twelve hours. The remaining extraction procedure followed as above.

DNA Amplification Protocols

Pipet tips with a filter barrier were used throughout this process to guard against contamination of the reactions. Negative controls were included during the DNA extractions and PCR reactions to detect contamination if it did occur. Each extracted DNA sample was diluted 1:10 in TE buffer, and 2 µl of that dilution was used in a 50 µl PCR reaction. Modified versions of the universal eukaryotic primers A and B (with the polylinkers removed) from Medlin et al. (1988) were used in the initial DNA amplifications of the 18S rRNA gene. Primer sequences are as follows: A (forward) 5'-AACCTGGTTGATCCTGCCAGT-3', B (reverse)-- 5'-TGATCCTTCTGCAGGTTACCTAC-3'. We found the 18S rRNA gene to be roughly 1800 bp in length in anthozoans. Thirty-five cycles of PCR were carried out using a Perkin Elmer Thermal Cycler 480. The DNA was denatured at 94°C for 45 seconds, the primers and template were annealed at 55°C for one minute, and the original DNA strand was extended at 72°C for 90 seconds. These 35 cycles were followed by a five minute

extension at 72°C. The product was visualized on a 1% agarose gel. There was always a visible product from the fresh or frozen tissues, and that product was prepared for cloning.

For those museum specimens that yielded no visible PCR product, a second PCR reaction was conducted using 1 µl of product from the initial PCR reaction as the template. The amplified negative control (no DNA was added to the tube) from the initial PCR reaction was included in the second reaction, using 1 µl of the original negative control as template. The primers used for the second PCR reaction were chosen to ensure that at least one primer annealed internally to the initial A and B primers. The internal primers were selected from a combination of universal eukaryotic primers and a set of octocoral-specific primers that were designed by me. The universal primers were the following: 373 (forward) 5'-GATTCCGGAGAGGGAGCCT-3' and 1200 (reverse) 5'-GGGCATCACAGACCTG-3' (Weekers et al. 1994), 514 (forward) 5'-GTGCCAGCMGCCGCGG-3', 1055 (forward) 5'-GGTGGTGCATGGCCG-3', and 1055 (reverse) 5'-CGGCCATGCACCACC-3' (Elwood et al. 1985), and 536 (reverse) 5'-WATTACCGCGGCKGCTG-3' (Lane et al. 1985). The octocoral-specific primers were designed to amplify anthozoan DNA, but not DNA from the potential contaminants. The octocoral-specific primers were designed from alignments of GenBank sequences of actinarians (found in Table 1), two fungi (*Cryptococcus neoformans*, Genbank accession #L05428, and *Bullera unica*, accession #D78330), potential epibionts from Mollusca and Crustacea (*Mytilus galloprovincialis*, accession #L33451, and *Stenocypris major*, accession #Z22850), and a zooxanthella symbiont (*Symbiodinium* sp., accession #M88509). Octocoral sequences derived from frozen tissue in this laboratory (Table 1) were verified as cnidarian through a BLAST search of GenBank and were also used in the primer design. Octocoral-specific primers were the following: 705 (forward) 5'-GGTCAGCCGTAAGGTTT-3', 705 (reverse) 5'-CATACCTTTCGGCTGACC-3', 900 (forward) 5'-GTTGGTTTTTTGAACCGAAG-3', 900 (reverse) 5'-

CTTCGGTTCTAGAAACCAAC-3', 1560 (reverse) 5'-GGTGAAGGAGTTACTCGATG-3'. PCR primer pairs were chosen to include at least one octocoral-specific primer, and to amplify the largest fragment possible from the archival specimens. Further details and the rationale behind this technique can be found in Chapter 2.

Determination of DNA Sequences

The final PCR product was cloned using the Original TA Cloning Kit (Invitrogen Corporation). The PCR product was ligated into the pCR 2.1 cloning vector, then transformed into a strain of INV α F' cells. The plasmid was isolated using the Wizard Miniprep DNA Purification Kit (Promega Corporation) and subsequently used as a template for cycle sequencing reactions, using the SequiTherm EXCEL Long-Read DNA Sequencing Kit-LC (Epicentre Technologies). DNA sequences were determined for both the forward and reverse strands of the gene. The reactions were run on a LI-COR 4000 DNA Sequencer, using infrared-labeled primers: M13 (forward) 5'-CACGACGTTGTAAACGAC-3', M13 (reverse) 5'-GAATAACAATTTACACAGG-3', 514 (forward) 5'-TCTGGTGCCAGCASC CGCGG-3', 536 (reverse) 5'-TGGWATTACCGCGGSTGCTG-3', 1055 (forward) 5'-GTGGTGGTGCATGGCCG-3', 1055 (reverse) 5'-AAGAACGGCCATGCACCAC-3'. The resulting images were interpreted using the BioImage gel reader program.

Sequence Analysis

DNA sequences were aligned first by eye, with consideration of secondary structure models, and with the alignment program Clustal W 1.6 (Thompson et al. 1994). Regions of uncertain alignment were eliminated from the final analyses (the nexus file is available upon request). In total, 1609 basepairs were used in the analyses, of which 247

out of 541 variable sites were parsimony-informative, and 294 were parsimony-uninformative.

All analyses were performed using test versions of PAUP* (ver. 4d61, 4d63) (Swofford 1996, betatest version). An initial distance analysis of the entire dataset was performed using a Kimura 2-parameter model. The likelihood scores were calculated for that tree using a variety of ML models, incorporating combinations of base-dependent rates of change with unequal base frequencies, a proportion of invariant sites, and substitutional rate heterogeneity. The purpose of this procedure was to identify the simplest model of evolution that was still accurate for this data set; the simpler model has a lower variance (Rzhetsky & Nei 1995) and is less computationally intensive. Using a Likelihood Ratio Test (LRT) similar to the one described by Huelsenbeck and Rannala (1997), the likelihood scores (L) of simpler models were compared to that of the most complex reference model (a general time-reversible model with among-site substitution heterogeneity): $LRT=2([- \ln L_{reference}] - [- \ln L_{alternative}])$. The values of the LRT are approximately Chi-square distributed, with the degrees of freedom equal to the difference in free parameters between the models being tested. Models for which the LRT score was greater than the Chi-squared critical value were rejected as not being sufficiently accurate.

Likelihood scores were also used to test specific phylogenetic hypotheses addressed by this study. Alternative evolutionary hypotheses were formed by manipulating tree topologies using the computer program MacClade (Maddison & Maddison 1992). The Kishino-Hasegawa (KH) Test (Kishino & Hasegawa 1989) within the PAUP* program was used to compare the likelihood scores of different topologies (i.e. evolutionary hypotheses). Trees were viewed using PAUP* and the free-ware program TreeView (Page 1996).

Results

The large number of taxa and lengths of each sequence included in these analyses made it unlikely that simple parsimony or distance methods would be sufficient for detecting the phylogenetic signal within this data set. When using subsets of the data, e.g. 23 out of the 47 taxa, bootstrap analyses of both parsimony and Kimura two-parameter distance methods gave trees with well-supported topology as defined by high bootstrap values. When the full data set was used, however, the placement of the Order Ceriantharia changed substantially, and the bootstrap values of the basal nodes dropped from 80-100% in the subset analysis to 50-60% and below with the full data set. The results from the LRT using the original Kimura 2-parameter tree showed that simple parsimony and distance models were insufficient to model the evolution of this gene, and that the appropriate model for the analysis of this data set was a general time-reversible model (GTR) with among-site heterogeneity. All simpler models were statistically inferior to the full GTR model for explaining this neighbor-joining tree given these data. The parameters estimated from the Kimura 2-parameter tree (Table 2) were used in a heuristic ML search consisting of five replicates, with random addition of sequences. The parameters were re-estimated using the most likely tree that was produced from that ML analysis, to insure there were no substantial changes. The most likely trees were found within the first replicate in all analyses conducted.

Seven species were tested as potential outgroups for these analyses: two species from Phylum Porifera, two species from Phylum Ctenophora, one species from Phylum Placozoa, and two other species from the Phylum Cnidaria, representing classes Hydrozoa and Cubozoa (Table 1). Species were tested individually and as a group to determine their effect on the overall tree topology. The major clades were present regardless of the taxa chosen for the outgroup, with the exception of *Trichoplax*. When *Trichoplax* was used as

Sequence Parameter	Value
Base frequencies	
A	0.257
C	0.206
G	0.275
T	0.262
Substitution R matrix	
A/C	0.9458
A/G	2.6447
A/T	0.7776
C/G	1.6166
C/T	4.9021
Proportion of invariant sites	0.3807
Gamma distribution	
α	0.5889

Table 2 Sequence parameter values calculated from the 18S rDNA used in the present study. Values used as input for maximum likelihood analyses. The R matrix contains base-specific substitution rates. The gamma distribution parameter α is the inverse of the coefficient of variation of the substitution rate.

the sole outgroup, the major clades which had been well-supported were broken up and the hexacorals were no longer monophyletic. Polyphyly of the Hexacorallia is inconsistent with traditional morphological taxonomy, and has not been shown by previous molecular phylogenies, and it is therefore likely that *Trichoplax* by itself is an inappropriate outgroup for these analyses. When all outgroups were used together, the general tree topology was once again as seen in the majority of the outgroup trials.

The positional stability of the outgroups was examined in addition to their overall effect on tree topology. Although the combined use of *Mnemiopsis leidyi* (ctenophore) and *Selaginopsis cornigera* (hydroid) as the only outgroups produced the expected tree topology, *M. leidyi* could then be moved to multiple positions on the topology, including to the ingroup, without reducing the likelihood score significantly (KH Test, $P < 0.05$). *Mnemiopsis leidyi* with *S. cornigeras* alone may be an inappropriate outgroup, since *M. leidyi* doesn't branch reliably at the root of the tree. When all outgroup species were used, the root became stable; the outgroup species could only be placed at the root or the adjoining basal node without reducing the likelihood score significantly. Monophyly of the ingroup could not be rejected. The final analyses for this data set were performed using all seven taxa as outgroups.

Parsimony and distance methods showed that the orders Actiniaria, Zoanthidea, Scleractinia, Antipatharia, and Ceriantharia, as well as the Subclass Octocorallia, constitute monophyletic groupings (data not shown). The Corallimorpharia do not appear to be monophyletic. Since the primary issues to be addressed here were the ordinal-level relationships within the Anthozoa, the topology and monophyly of the Scleractinia and the Octocorallia were fixed for the ML analyses to decrease computational complexity (i.e. effectively reducing the number of taxa from 41 to 25) and to concentrate the analytical efforts on the above issues. One most likely tree was produced from a ML analysis (Fig.

2). The orders that were not fixed retained the monophyly found in distance and parsimony analyses.

The most likely tree (Fig. 2) indicated that the Subclass Ceriantipatharia is not a monophyletic grouping. The Order Ceriantharia was basal to the Hexacorallia, and the Order Antipatharia fell within the Subclass Hexacorallia, as a sister group to the Order Zoanthidea. The two species of the Ceriantharia clustered consistently with each other, but the placement of the Ceriantharia branch was not stable. Although the most likely tree placed the Ceriantharia in an ancestral position relative to the Hexacorallia, the cerianthids could then be placed in numerous positions throughout the Actiniaria, Antipatharia, within the Octocorallia, and at the base of most subclades without reducing the likelihood of the tree significantly (KH Test, $P > 0.05$) (Fig. 3). The cerianthids appear to be highly divergent from the other Anthozoa, but they have evolved in such a way that the phylogenetic signal from their sequences could not indicate their specific phylogenetic affinities within the Anthozoa. Their position could not be placed reliably using nuclear 18S rRNA data. The two cerianthid species were eliminated from further analyses in order to reduce the computational noise resulting from their sequences.

The computational complexity of the analyses was reduced further by fixing the relationships within the Actiniaria, the Antipatharia, the Zoanthidea, the Scleractinia, and the Octocorallia, as they were found in parsimony analyses. This effectively reduced the number of taxa in the analysis from 45 to 14. This approach was justified based on the solid bootstrap support for monophyly, as seen in a ML-calculated distance analysis (Fig. 4), as well as results from parsimony and ML analyses (data not shown). The fixation of these nodes allowed the ML algorithm to concentrate on the relationship of the basal nodes, which represent the relationships among the primary clades.

A single most likely tree was found from the subsequent ML analysis (Fig. 5). The Subclass Octocorallia formed a sister clade to the Hexacorallia. Within the hexacoral clade,

Fig. 2 The most likely tree produced from maximum likelihood analyses of the Anthozoa, with topologies of the octocoral clade and scleractinian clade fixed (circled) (-LN likelihood = 11919.609). The relative positions of the orders within the Hexacorallia and Ceriantipatharia (Scleractinia, Corallimorpharia, Antipatharia, Zoanthidea, Actiniaria, and Ceriantharia) are interchangeable without reducing the likelihood of the tree significantly (KH Test, $P < 0.05$). Horizontal branch length reflects genetic distance among taxa. Outgroup genera are *Tetilla*, *Scypha*, *Beroe*, *Mnemiopsis*, *Trichoplax*, *Tripedalia* and *Selaginopsis*.

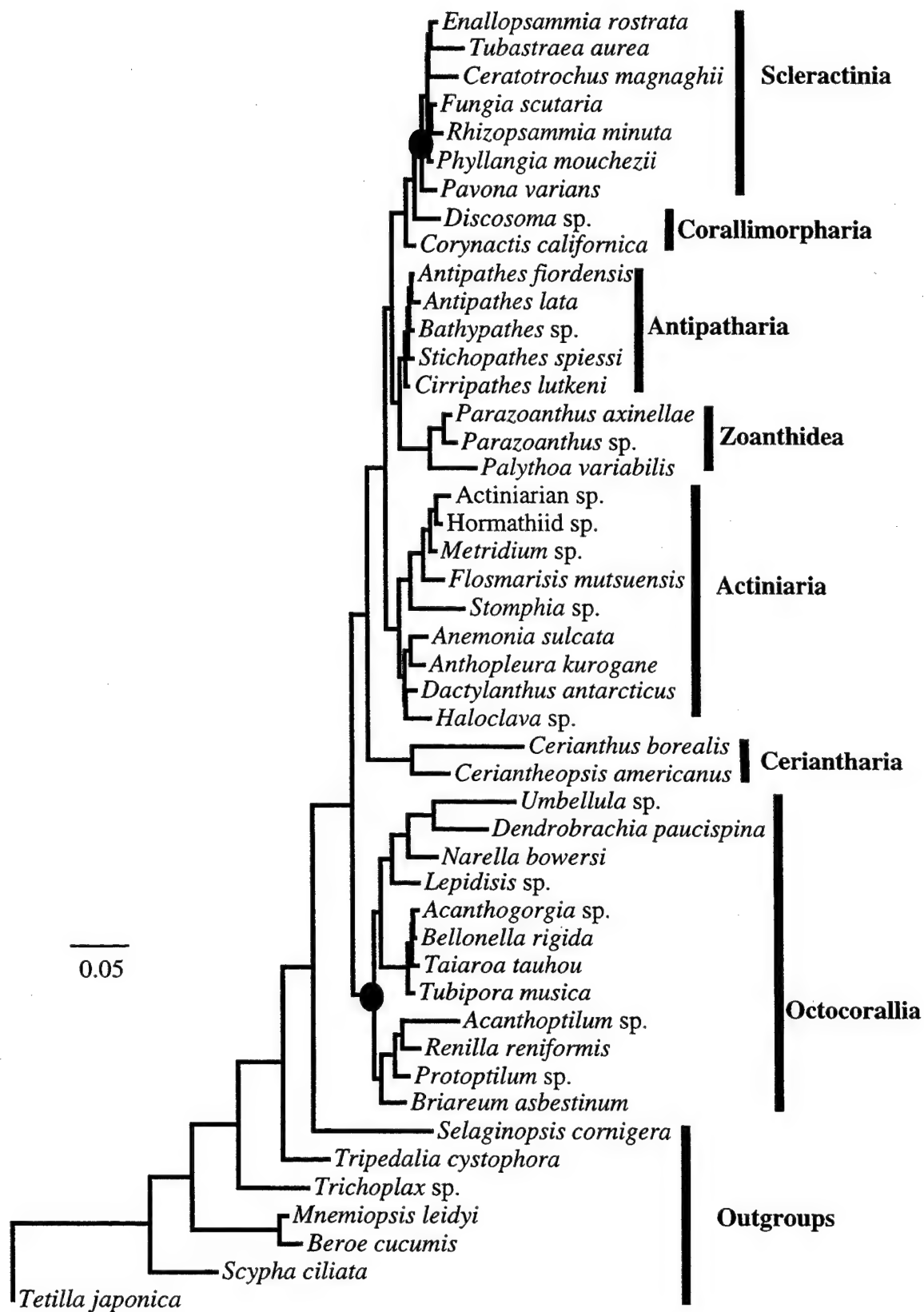


Fig. 3 Possible placements of the Ceriantharia that do not reduce the likelihood of the tree. The X's mark the positions where one or both species of the Order Ceriantharia can be placed without a significant reduction of the likelihood score of the tree (KH Test, $P < 0.05$). Horizontal branch length does not reflect genetic distance. Outgroup genera are *Tetilla*, *Scypha*, *Beroe*, *Mnemiopsis*, *Trichoplax*, *Tripedalia* and *Selaginopsis*.

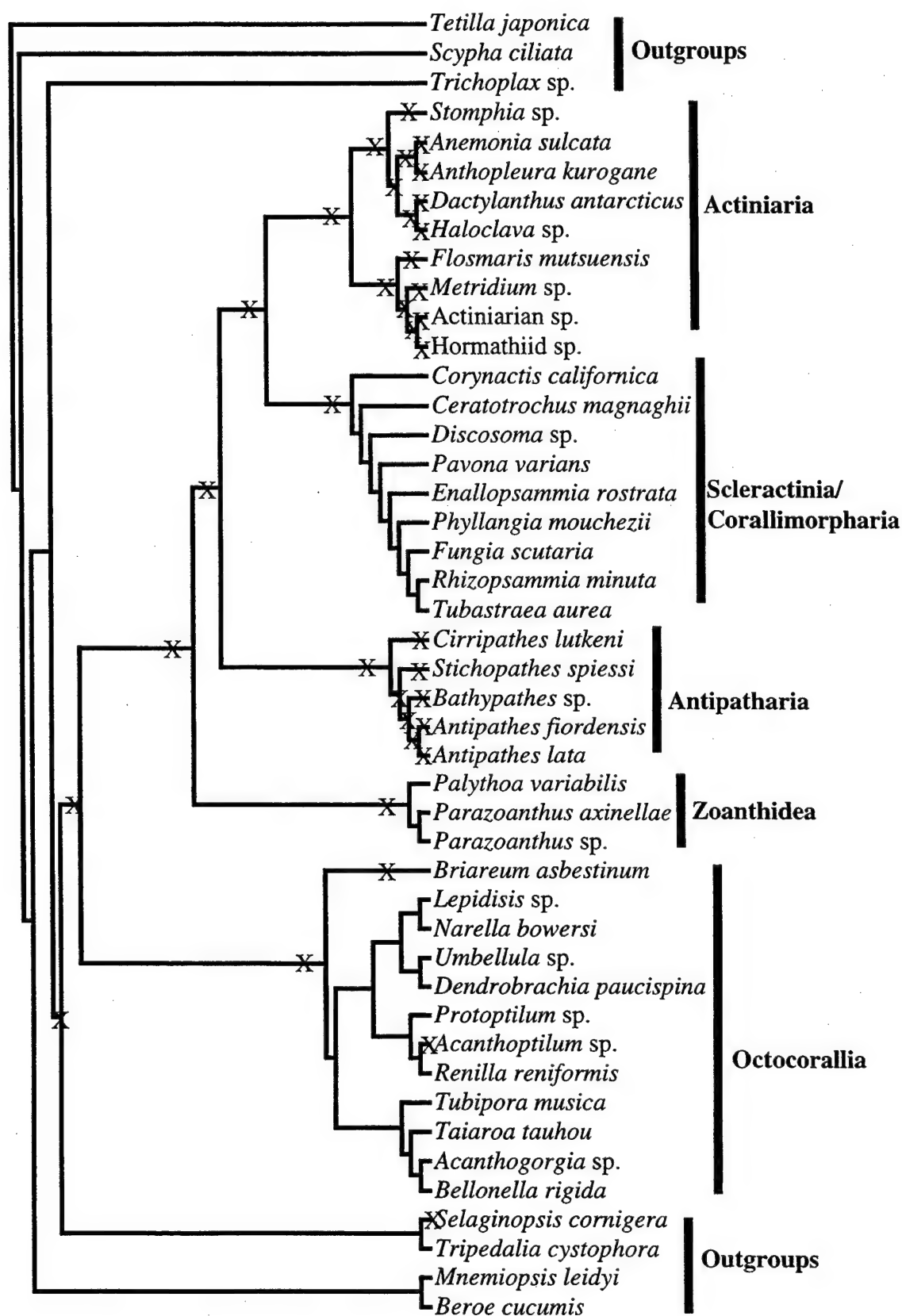


Fig. 4 **Bootstrap analysis using distances calculated from maximum likelihood parameters, showing support for monophyly of the ordinal nodes. No taxa were fixed for this computation. Horizontal branch length reflects genetic distance among taxa.**

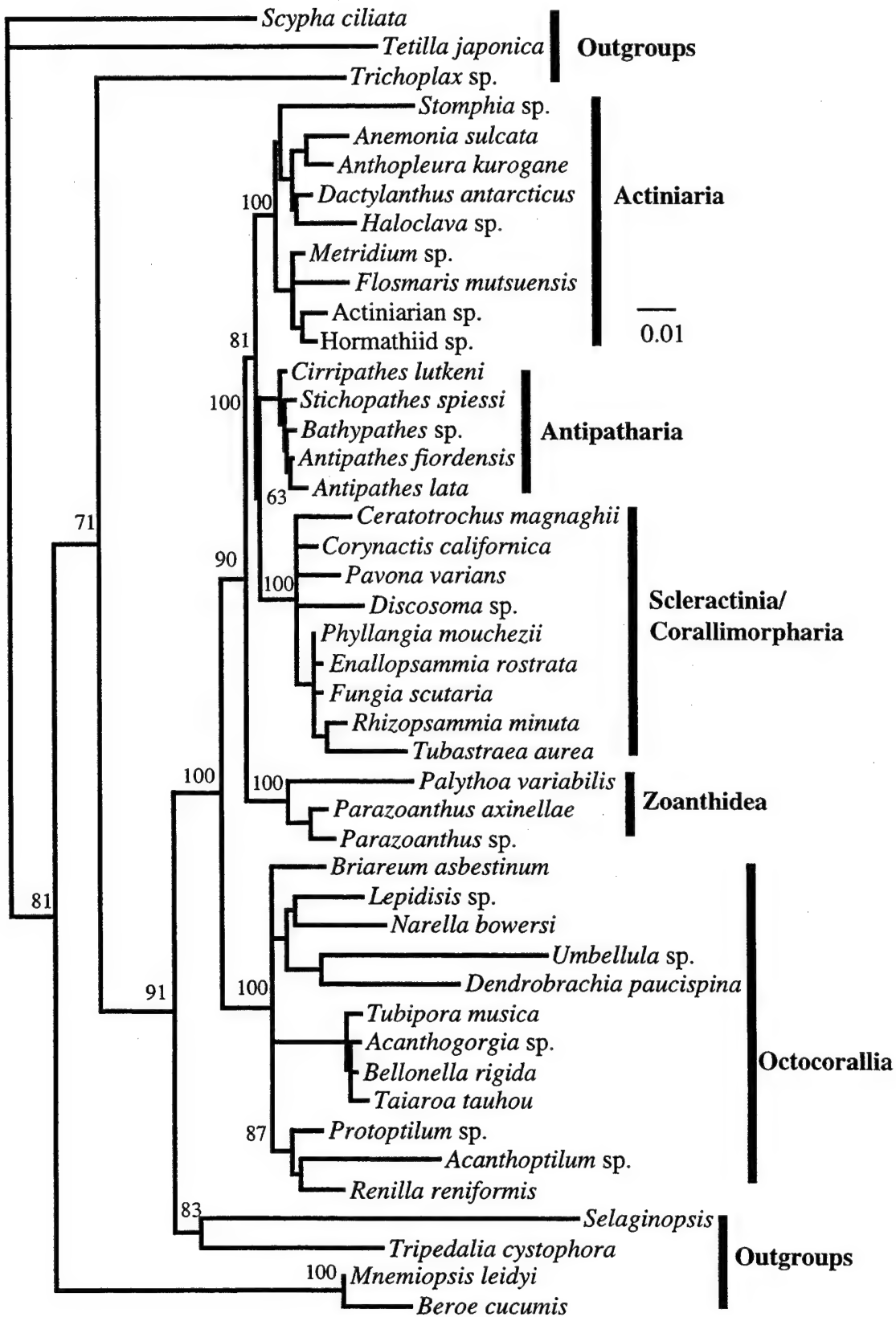
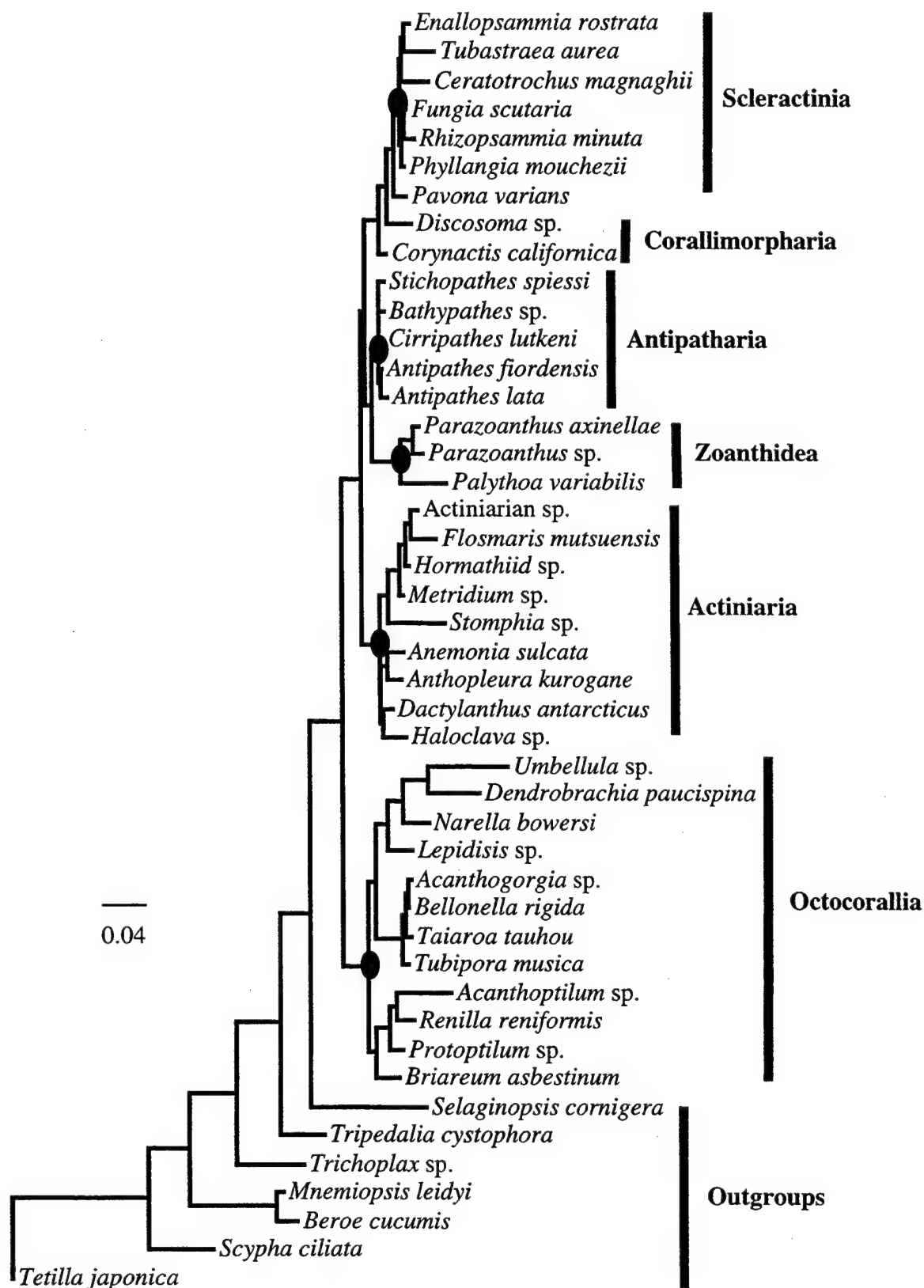


Fig. 5 Most likely tree produced from maximum likelihood analyses, showing the most basal relationships present among the Hexacorallia (-Ln likelihood = 11072.545). The topologies of the Actiniaria, Antipatharia, Scleractinia, Zoanthidea, and Octocorallia were fixed to simplify computational complexity (circled). Horizontal branch length reflects genetic distance among taxa.



the Actiniaria were most basal. The Antipatharia formed a sister clade to the Zoanthidea. The Corallimorpharia were polyphyletic, and basal to the Scleractinia. The topology of ordinal clades within the Hexacorallia can be varied without reducing the likelihood of the tree significantly (KH Test, $P < 0.05$).

The 18S rRNA gene contained sufficient phylogenetic signal to suggest relationships for both the ptychodactarian species *Dactylanthus antarcticus* and the putative antipatharian *Dendrobrachia paucispina*. Neither of these species was constrained for the initial analyses, as I wanted to determine their position relative to the other Anthozoa. The ptychodactarian *Dactylanthus* was firmly allied with the Order Actiniaria. A separate ML analysis was conducted including only *Dactylanthus*, the Actiniaria, and a subset of the outgroup species. New ML parameters were calculated to reflect the change in taxonomic sampling. The most likely tree (Fig. 6) placed *Dactylanthus* with the species *Haloclava* sp..

Dendrobrachia paucispina was placed clearly within the octocoral clade, and branched with the species *Umbellula* sp. and *Narella bowersi*. All other positions for *Dendrobrachia* on this tree reduced the likelihood significantly (KH Test, $P < 0.05$).

Discussion

These molecular analyses support the morphological division between the Subclass Octocorallia and the other Anthozoa, which was based primarily on mesentery and tentacle structure. The octocorals' octamerous mesenteries and pinnately-branched tentacles are unique to the Anthozoa, as are their mesenchymal skeletal spicules. The octocorals also share a single type of nematocyst (basitrichs) which are rare or absent in the Actiniaria (Picken & Skaer 1966, Schmidt 1974). The remaining species within the Anthozoa, which comprise the early Subclass Hexacorallia, are more diverse in their morphology. They are characterized by simple, unbranched tentacles, and generally by six or more pairs of

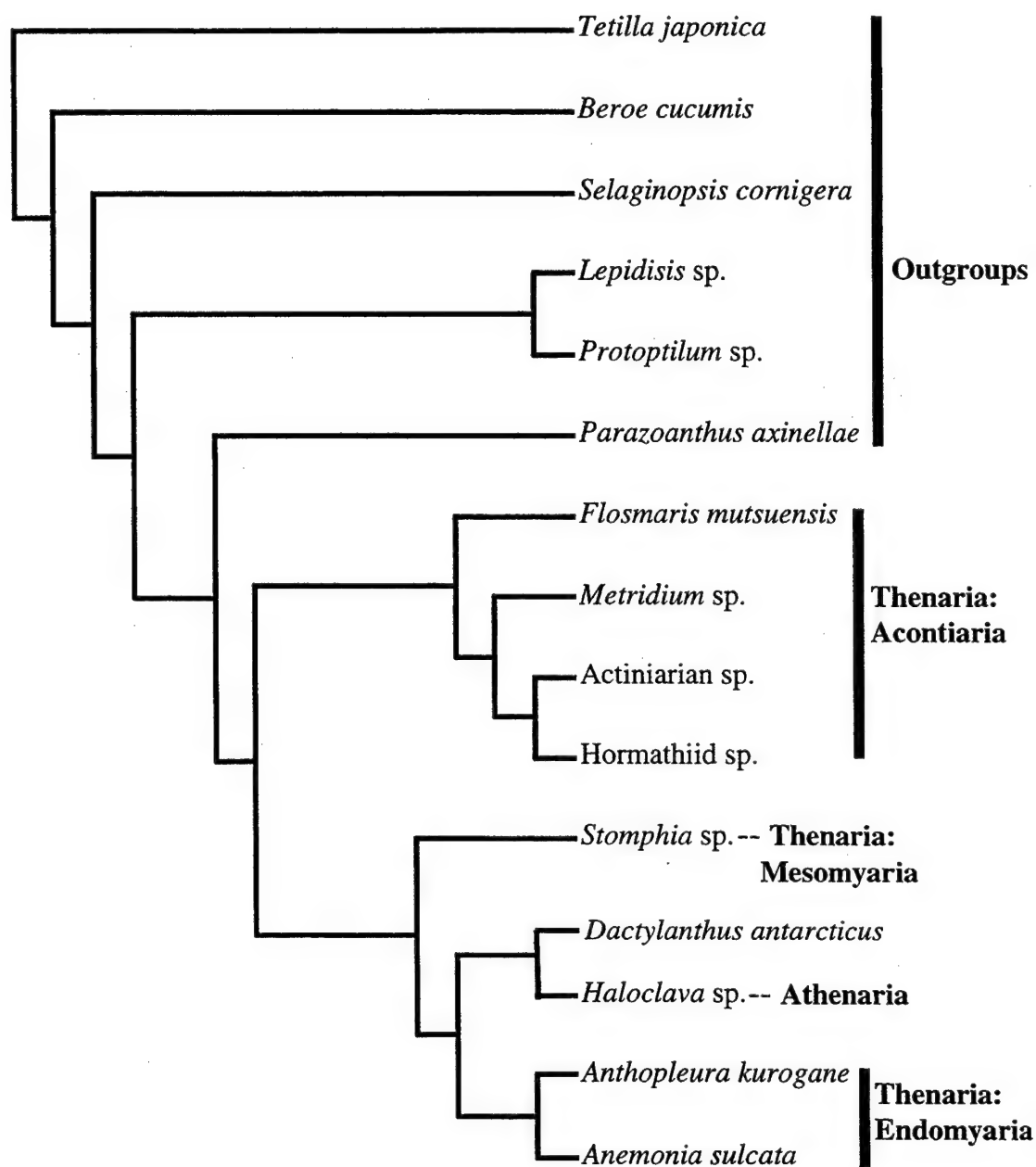


Fig. 6 Maximum likelihood analysis of Actiniaria and Ptychodactiaria (*Dactylanthus antarcticus*) (-Ln likelihood = 6094.807). *Dactylanthus* does not appear to constitute a separate order. It is most closely allied with the tribe Athenaria (Suborder Nynantheae). Tribe Athenaria and *Dactylanthus* branch within the clade of the Tribe Thenaria (Suborder Nynantheae).

mesenteries. Their skeleton (if present) may be composed of calcium carbonate or chitin, but never contains free sclerites (Wells & Hill 1956a).

Molecular information from three different ribosomal genes have indicated two potential phylogenetic positions for the Ceriantharia within the Anthozoa. France et al. (1996) examined the mitochondrial 16S rRNA gene (550-900 bp from 29 species), and found the cerianthids were ancestral to the remaining hexacorals (Fig. 1a). A combination of 16S mtDNA and 18S rDNA sequences also found the cerianthids to be basal to the hexacorals (Bridge et al. 1995). A third study using 800 bp of the 18S rDNA from 13 species (Song & Won 1997) placed the cerianthid species as ancestral to all of the Anthozoa (Fig. 1b), as did a fourth study using 225 bp of 28S rDNA sequence from 22 species (Chen et al. 1995) (Fig. 1c).

The most likely tree generated from the 18S rDNA sequences in the present study did not support the alliance of the orders Ceriantharia and Antipatharia within the Subclass Ceriantipatharia (Fig. 1). Similar conclusions were found previously by France et al. (1996) and Song/Won (1997). The Ceriantharia appeared to be ancestral to the Hexacorallia, and the Antipatharia fell within the Hexacorallia. These analyses could not, however, identify the exact position of the Ceriantharia with statistical certainty (Fig. 3). The cerianthid species could be placed in multiple positions on the tree without reducing the likelihood of the tree significantly. These analyses of 18S sequence information were unable to establish the phylogenetic history of the Ceriantharia reliably.

I was able to clearly establish the affinities of the Order Antipatharia within the Subclass Hexacorallia from my analyses. Sequence analyses in the past have had incomplete sampling for the determination of the phylogenetic affinities of the Antipatharia. France et al. (1996) and Song and Won (1997) found the Antipatharia to be a sister clade to the Actiniaria, but neither study included zoanthid species in their analyses. The most likely tree from my analyses indicated the Antipatharia form a sister clade to the Zoanthidea.

Moving the Antipatharia to branch with the Actiniaria, however, did not make the tree significantly less likely. A phylogenetic association between the Antipatharia and either the Zoanthidea or the Actiniaria was supported from these analyses.

The most likely tree generated from my 18S sequence data suggested that the Corallimorpharia were not monophyletic, and they exhibited a closer affinity to the Scleractinia than the Actiniaria (Fig. 5). This result agreed with the France et al. (1996) study (Fig. 1a), but not with Chen et al. (1995) (Fig. 1c). The latter showed the Actiniaria to be a polyphyletic group comprised of two clades, with species of Corallimorpharia branching with both clades of actinarian species. These two actinarian/corallimorpharian clades were distinct from the monophyletic clade of scleractinians. My most likely tree indicated that both the Actiniaria and the Scleractinia were monophyletic, and the Corallimorpharia branched basally to the Scleractinia. The Corallimorpharia could be moved throughout the scleractinian clade, however, without reducing the likelihood of the tree (KH Test, $P < 0.05$). The likelihood of the tree was also not reduced significantly if the corallimorpharian species were forced to monophyly. The close phylogenetic association of the Corallimorpharia and the Scleractinia is reminiscent of the taxon Madreporaria (Stephenson 1921), in which were combined the Scleractinia and the Corallimorpharia. The close genetic affinity of the Scleractinia and the Corallimorpharia suggests that the morphological characters of mesentery structure, which is similar between the two groups, may be more reflective of evolutionary associations than the presence or absence of a skeleton.

The branching pattern of the Actiniaria in the present study differed from that found by Chen et al. (1995). I included representatives of the actinarian taxa that branched with the Corallimorphidae in the 28S tree, but they fell with the other Actiniaria and not with the Scleractinia according to my 18S data. The polyphyly of the Actiniaria found by Chen et al. may have been a result of the short sequence length used for their analyses.

My taxonomic sampling is limited for the Corallimorpharia, but I chose species that represented both of the major morphotypes within the order: *Discosoma* is of the tropical, plate-like variety, and *Corynactis* is of the temperate, polyp-like variety. This sampling scheme is clearly insufficient for indicating relationships among the families of the Corallimorpharia, and as I have shown the 18S rRNA gene may also be inadequate for distinguishing the relationships within this group. The close relationship between the Scleractinia and the Corallimorpharia is evident, but the phylogenetic information present in these 18S sequences is insufficient to establish the specific relationships among these two groups.

My 18S rDNA data indicated that the Ptychodactiaria have strong connections to the Actiniaria. These data do not support the establishment of a separate order, although the possibility a separate order is warranted cannot be strongly rejected. I conducted an ML analysis on the Actiniaria and *Dactylanthus* alone, with a selection of species as the outgroup, and new parameters appropriate to these taxa. My analyses indicated good agreement between the 18S-generated relationships and traditional placement of *Dactylanthus* within the Actiniaria (Fig. 6). The subtribes each constituted monophyletic groupings. The Tribe Anthenaria, represented here by a single species, appeared to be derived from the Tribe Thenaria. The most likely tree from my analysis of this subset of species showed a sister-taxon relationship between *Dactylanthus* and *Haloclava*, of the Tribe Athenaria. However, *Dactylanthus* could be moved throughout the Endomyaria/Athenaria clade, or to a position basal to the Acontiaria, or basal to the Actiniaria as a whole without reducing the likelihood significantly (KH Test, $P < 0.05$). This sampling scheme also lacks representatives of the two remaining suborders within the Actiniaria (Suborder Protantheae and Suborder Endocoelanthaeae). Thus, although the association of *Dactylanthus* with the Actiniaria is clear, its exact position within the Actiniaria remains unresolved.

These 18S sequence information confirmed the divergence between *Dendrobrachia* and the Antipatharia, the order in which it was originally placed (Fig. 5). *Dendrobrachia paucispina* fell securely within the octocorals, specifically with two species of the Suborder Holaxonia (*Lepidisis* sp. and *Narella bowersi*), and the unusual pennatulacean *Umbellula* sp.. Opresko and Bayer (1991) stated that *Dendrobrachia* is clearly an octocoral, based on its definitively gorgonian polyp structure. Although the genus has a spiny, chitinous skeleton and lacks free sclerites, these are the only characters shared with the Antipatharia. Opresko and Bayer (1991) suggested that *Dendrobrachia* may be associated with the Family Chrysogorgia, which is also within the Suborder Holaxonia, and my analyses were consistent with this hypothesis. It appears the skeletal composition is less important than polyp morphology for indicating taxonomic affinities for this group. A more comprehensive discussion of the phylogenetic position of *D. paucispina* appears in Chapter 4.

Conclusions

These analyses of 18S rDNA data indicate that the three-subclass system as it exists currently is not reflective of the evolutionary history of the Anthozoa. A three-subclass system may indeed be accurate, with the Ceriantharia designated a subclass with the Hexacorallia and the Octocorallia. The orders Ceriantharia and Antipatharia, united currently within the Subclass Ceriantipatharia, however, are genetically disparate groups. My data support the hypothesis that the Order Ceriantharia is ancestral to the Subclass Hexacorallia, while the Antipatharia are sister-taxa to the Zoanthidea and are highly derived within the Hexacorallia (as from Brook 1889, Hickson 1906, and Hadzi 1963). Although the most likely tree supports this conclusion, I cannot reject alternate hypotheses for the placement of the Ceriantharia as ancestral to the Anthozoa (as shown by Song & Won 1997). The phylogenetic affinities of the Ceriantharia could not be determined reliably

using these data. It is clear that they are highly divergent from the remaining Anthozoa, but their ancestry remains uncertain.

The Subclass Octocorallia, as well as the orders Actiniaria, Scleractinia, Zoanthidea, Ceriantharia, and Antipatharia were each well-supported monophyletic groupings. The Order Corallimorpharia did not appear to be monophyletic, however, and my results show that its affinities lie with the scleractinians. The internal mesentery structure appears to be a better character than the presence of a skeleton for indicating evolutionary patterns of the Corallimorpharia. This was suggested with the historical association of the Scleractinia and the Corallimorpharia within the Madreporaria, separate from the Actiniaria. Sequence information from genes with higher levels of divergence, as well as from additional species of Corallimorpharia, will likely be necessary to establish the relationships between the Corallimorpharia and the Scleractinia.

The phylogenetic affinities of the Ptychodactiaria and the genus *Dendrobrachia*, both of which have been equivocal, were established using 18S sequence information. The ptychodactarian *Dactylanthus* was not genetically distinct from the Actiniaria, a result which is consistent with their original familial designation within the Actiniaria. My sequence information suggested an association of *Dactylanthus* with the Tribe Athenaria, but a more specific classification will require further sampling within the Actiniaria.

Sequence information showed that *Dendrobrachia* was allied with the Octocorallia, as has been predicted from recent morphological work. My analyses placed *Dendrobrachia* closest to members of the Suborder Scleraxonia and the pennatulacean *Umbellula*. Again, further analyses including a more complete representation of the octocorals will be necessary to firmly establish its phylogenetic position within the subclass.

This study has provided the most complete analysis of relationships across the Anthozoa to date. I have used the full 18S rDNA sequence for 38 species across all extant orders, and have conducted Maximum Likelihood techniques employing appropriate

models of evolution for this gene. Nuclear 18S sequence data have clearly shown the major divisions within the Anthozoa, and suggested phylogenetic affinities for species that had been enigmatic. Sequence information suggests that skeletal properties may not be a defining character indicating evolutionary relationships within the Anthozoa. Not all of the alternate hypotheses arising from other molecular studies can be supported or refuted with these data. Sequence information from additional genes and additional species will be necessary to establish the specific relationships investigated here.

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Chapter 4

Phylogenetic Relationships within the Subclass Octocorallia (Phylum Cnidaria: Class Anthozoa), Based on Nuclear 18S rRNA Sequence Information

Abstract

Historically, the Subclass Octocorallia was divided into seven orders: Helioporacea (Coenothecalia), Protoalcyonaria, Stolonifera, Telestacea, Alcyonacea, Gorgonacea, and Pennatulacea. It has been argued that this arrangement exaggerates the amount of variability present among the species of the Octocorallia. The current taxonomy recognizes the two orders of Helioporacea (blue corals) and Pennatulacea (sea pens), and assembles the remaining species into a third order, Alcyonacea. The species within the Alcyonacea exhibit a gradual continuum of morphological forms, making it difficult to establish concrete divisions among them. The subordinal divisions within the Alcyonacea correspond loosely to the traditional ordinal divisions. In this study I used molecular techniques to address the validity of the historical ordinal divisions and the current subordinal divisions within the Subclass Octocorallia. I also explore the phylogenetic affinities of the species *Dendrobrachia paucispina*, which was originally classified in the Order Antipatharia (Subclass Ceriantipatharia). Polyp structure indicates a closer affinity between *Dendrobrachia* and the Subclass Octocorallia. I have determined the nuclear 18S rRNA sequences for 41 species of octocorals, and use these to construct a molecular phylogeny of the subclass. I utilize Maximum Likelihood techniques, employing a realistic model of evolution for these species and this data set. The most likely trees from these sequence data indicate three clades, and do not support the morphological taxonomy of the Octocorallia. The Order Pennatulacea is the most cohesive group within the subclass, but is not monophyletic. One clade is undifferentiated and contains half of the species in this analysis. The third clade contains members from three suborders of the Alcyonacea, and one member of the Pennatulacea. These data cannot be used to distinguish among the branching order of these three clades. The morphological character of dimorphism (the presence of both autozooids and siphonozooids within a single colony) corresponds

loosely with the topology of the most likely trees, and the monophyly of dimorphism cannot be rejected from these data. The species *Dendrobrachia paucispina* has a close affinity with the genera *Corallium* and *Paragorgia* (Alcyonacea: Scleraxonia), although its morphology suggests it is more similar to the genus *Chrysogorgia* (Alcyonacea: Holaxonia). The genetic divergence found within genera is approximately equivalent to that found in other invertebrates, but the divergence found within families is greater in the octocorals than in other invertebrates. This difference may reflect the inappropriate inclusion of evolutionarily divergent genera within octocorallian families. I have employed appropriate evolutionary models for maximum likelihood analyses in this study, utilizing complete 18S rDNA sequences from the majority of families within the Octocorallia. Many of the relationships within the Octocorallia, however, remain ambiguous.

Introduction

The Subclass Octocorallia (Phylum Cnidaria) contains many well-known species of invertebrates, including soft corals, gorgonians, sea pens, and blue corals. They are exclusively polyp-shaped, and constitute a well-defined morphologic group. Several characters unite them: nematocyst structure, tentacle number and structure, and the number and structure of their mesenteries (divisions within the gastrovascular cavity). The octocorals are relatively simple taxa morphologically, however, with few remaining characters with which to distinguish taxonomic groupings within this subclass. They can be solitary or colonial, and may possess a skeleton consisting of a calcium and/or chitinous axis and extra-skeletal calcium-carbonate spicules. The axial structure and the spicular size, structure and arrangement are particularly useful for distinguishing among groups at all taxonomic levels within the octocorals. Other characters which are used to determine taxonomic groupings include the distribution of polyps and the budding pattern of new polyps, monomorphic vs. dimorphic colonies (autozooids and siphonozooids within the

same colony), and the nature and arrangement of the calcium-carbonate spicules (Bayer 1956). The taxonomic characters available are so few that entire groups may have been defined based solely on a single characteristic (Hickson 1930). The fossil record for these species is also incomplete, contributing little towards the reconstruction of their evolution.

The traditional taxonomy for the Octocorallia divides the subclass into seven orders: Helioporacea (Coenothecalia), Protoalcyonaria, Stolonifera, Telestacea, Alcyonacea, Gorgonacea, and Pennatulacea (Fig. 1A). Morphology, however, distinguishes only two unique groups within the Octocorallia: the orders Helioporacea (blue corals) and Pennatulacea (sea pens) (Bayer 1956, Bayer 1973). The Helioporacea lack spicules, and form a massive crystalline aragonite skeleton, similar to some Hydrozoa and Scleractinia. The Pennatulacea are colonial and are dimorphic. The primary axial polyp is elongated and anchored into soft sediment, and the secondary autozooids branch from these primary polyps. Pennatulaceans are the most advanced in terms of their colonial complexity, functional specialization of zooids, and colonial integration (Bayer 1973).

The remaining species within the subclass are less easily classified based on morphology. One group of families (Ellisellidae, Ifalukellidae, Chrysogorgiidae, and Primnoidae) within the original Order Gorgonacea (Suborder Holaxonia) is clearly distinct from the remaining alcyonaceans based on its complete lack of chambered axial medulla. These families are sometimes grouped together as the 'restricted Holaxonia' (Bayer 1981). Firm divisions among the remaining species cannot be made on the basis of morphology. All degrees of colonial organization and skeletal form occur in a nearly uninterrupted continuum, and all of the major body plans are linked by intermediate forms that make strict morphological divisions difficult (Bayer 1973, Bayer 1981).

Although traditional taxonomy recognizes seven distinct orders, several species form morphological links between these groups: species of *Telestula* that were originally assigned with *Clavularia* link the Stolonifera and the Telestacea; *Protodendron repens* and

Subclass Octocorallia
Order Helioporacea
Order Protoalcyonaria
Order Stolonifera
Order Telestacea
Order Alcyonacea
Order Gorgonacea
Order Pennatulacea

A

Subclass Octocorallia
Order Helioporacea
Order Alcyonacea
 Suborder Protoalcyonaria
 Suborder Stolonifera
 Suborder Alcyoniina
 Suborder Scleraxonia
 Suborder Holaxonia
Order Pennatulacea

B

Fig. 1 Taxonomic classifications of the Subclass Octocorallia.
A) Traditional classifications, e.g. Deichmann (1936), Hyman (1940).
B) Revised classifications, from Bayer (1981).

Maasella radicans combine attributes of the Stolonifera and the Alcyonacea; the families Paragorgiidae and Briareidae have been assigned alternately to the Alcyonacea and Gorgonacea; species of *Keroeides*, *Lignella*, and *Ideogorgia* exhibit both the proteinaceous cross-chambered axis of the Holaxonia and the axial sclerites typical of the Scleraxonia (Bayer 1981). Bayer (1981) suggested that the amount of variation among these species was insufficient to warrant distinction at the ordinal level, and revised the classification accordingly. The orders Helioporacea and Pennatulacea were retained, and the remaining groups were established as suborders (listed with increasing level of colonial complexity) within the Order Alcyonacea (Fig. 1B). The remainder of the present paper will use Bayer's classification system as the reference.

The origins of the Octocorallia and their subsequent evolution remain to be determined, although the ancestral octocoral was likely solitary (Bayer 1973). The Pennatulacea are thought to be a very old group, however, although their precise origins are unclear. Pennatulacean-like species have been discovered from the Precambrian, preceding the appearance of any known gorgonian species (Bayer 1955, Bayer 1973). The Pennatulacea share morphological similarities with a number of alcyonacean species. Hickson (1916) suggested that the pennatulid Family Veretillidae may be the most primitive of the sea pens, and noted a similarity between the veretillids and a species of *Sarcophytum*, renamed *Anthomastus* (Alcyonacea: Alcyoniidae). The pennatulacean axial structure also has similarities to those of the gorgonian families Ellisellidae and Isididae, in patterns of chitinous and calcareous material extending outward from a calcareous core (Bayer 1955). Alternatively, the pennatulaceans may have arisen from a *Telesto*-like ancestor, the colony morphology of which superficially resembles that of the Pennatulacea (Bayer 1956, Bayer 1973). In 1900, Bourne proposed uniting the Pennatulacea and the Telestacea into a single group (cited in Hickson 1916). The lack of any known

morphological intermediates between the Pennatulacea and the other members of the subclass makes it difficult to establish evolutionary pathways.

In this study I utilized 18S rRNA molecular sequences to examine phylogenetic relationships within the Subclass Octocorallia. Molecular sequences can be used as additional characters, and can be useful for suggesting relationships among taxa with few morphological characters. The nuclear 18S rRNA gene has been useful in addressing questions on a variety of evolutionary scales, including relationships at the ordinal level, (e.g. Kelly-Borges et al. 1991, Hay et al. 1995) and the family level (e.g. Kuznedelov & Timoshkin 1993, Fitch et al. 1995).

The present phylogenetic analyses were based on complete 18S rDNA sequences from 41 species of octocorals, plus partial sequences for three additional species. I utilized Maximum Likelihood (ML) techniques, employing an evolutionary model applicable to the gene and organisms included in this study (Swofford et al. 1996, Huelsenbeck & Crandall 1997). It is particularly important to use realistic evolutionary algorithms when including this many taxa and basepairs. The amount of error introduced into an analysis through the use of inappropriate models may overwhelm the phylogenetic information contained in the data set (DeSalle et al. 1994, Rzhetsky & Nei 1995).

The specific goals of this study were to examine the primary phylogenetic groupings within the Subclass Octocorallia. I explored the genetic divisions within the Subclass Octocorallia with three primary inquiries: 1) is there molecular support for the either the historical ordinal divisions, or the subordinal divisions as they stand today, 2) is there genetic evidence for distinct divisions for the Helioporacea and Pennatulacea, and 3) is there any support for the ancestral nature of the Pennatulacea? The levels of genetic differentiation measured among groups was compared between orders within the Subclass Octocorallia and the Subclass Hexacorallia (Phylum Cnidaria). The hexacorals are likely appropriate models because they are closely related species, and their orders are fairly well

defined both on a morphological and a molecular level (Berntson et al. in prep). Traditional morphological characters were compared phylogenetic groupings found to evaluate which ones appeared to provide the most taxonomic information.

The final portion of this study examined the phylogenetic affinities of the intriguing species *Dendrobrachia paucispina*. This genus was originally classified with the Order Antipatharia (black corals) based on its chitinous, spiny axis and its lack of sclerites. A thorough examination of the polyp structure was not possible at that time due to poor sample preservation (Brook 1889). Even without information on polyp morphology, Brook (1889) noted similarities in the axial structure between *Dendrobrachia* and the Order Gorgonacea, but *Dendrobrachia* was placed with the Antipatharia because it lacked free sclerites. Recent morphological studies of this genus have determined that the polyp structure places *Dendrobrachia* unequivocally within the Subclass Octocorallia, specifically with members of the Family Chrysogorgiidae (Alcyonacea: Holaxonia) (Opresko & Bayer 1991). Our previous work with 18S rDNA sequence information (Berntson et al. in prep) suggested that *Dendrobrachia* has phylogenetic affinities with the octocorals, although our sampling at that time was insufficient to suggest its familial position. The present study provides sequences from all of the ordinal and subordinal groups within the Octocorallia, including 22 of the 30 extant families of Alcyonacea. This level of sampling was designed to determine whether *Dendrobrachia* was most closely related to the Chrysogorgiidae as predicted from morphology.

Methods

Specimens

The species used in this study (Table 1) were obtained from several sources. Forty sequences were determined for this work, and 13 additional sequences (including outgroups) were taken from Genbank. Ten of the octocoral specimens and the specimen of

Table 1 Specimens used in phylogenetic analyses. Species of Placozoa, Porifera, Ctenophora, Hydrozoa and Cubozoa were used as outgroups. ID# refers to museum accession numbers or to collection number. Specimen sources are as follows: AJ, courtesy of Ardis Johnston and the Harvard Museum of Comparative Zoology; Bishop Seamount, 18.8°N 159.1°W; CG courtesy of Constance Gramlich, University of California, San Diego; Cross Seamount, 18.7°N 158.3°W; Fieberling Guyot, 32.1°N 127.8°W; Pensacola Seamount, 18.3°N 157.3°W; NMNH, provided by Dr. Frederick Bayer, National Museum of Natural History, Smithsonian; TG, courtesy of Tamar Goulet, State University of New York, Buffalo.

Species	Source	ID #	Genbank Acc. #
Phylum Porifera			
Class Calcarea			
<i>Scypha ciliata</i>	Genbank		D15066
Class Demospongiae			
<i>Tetilla japonica</i>	Genbank		D15067
Phylum Ctenophora			
<i>Mnemiopsis leidyi</i>	Genbank		L10826
<i>Beroe cucumis</i>	Genbank		D15068
Phylum Placozoa			
<i>Trichoplax</i> sp.	Genbank		Z22783
Phylum Cnidaria			
Class Hydrozoa			
<i>Selaginopsis cornigera</i>	Genbank		Z92899
Class Cubozoa			
<i>Tripedalia cystophora</i>	Genbank		L10829
Class Anthozoa			
Subclass Hexacorallia			
Order Actinaria			
Suborder Nynanthea			
Tribe Thenaria			
Subtribe Endomyaria			
Fam. Actiniidae			
<i>Anemonia sulcata</i>	Genbank		X53498
Subtribe Acontaria			
Fam. Hormathiidae			
Hormathiid sp.	Fieberling Guyot, 640 m	AD2294-2	AF052890
Subclass Octocorallia			
Order Helioporacea			
Family Helioporidae			
<i>Helipora coerulea</i> (partial)	Arut Island, Indonesia, 1 m ^{NMNH}	79529	AF052923

Order Alcyonacea			
Suborder Protoalcyonaria			
Family Taiaroidae			
<i>Taiaroa tauhou</i>	Otago Peninsula, NZ, 420-320 m ^{NMNH}	54271	AF052908
Suborder Stolonifera			
Family Clavulariidae			
<i>Clavularia</i> sp.	Mission Bay, CA, 8 m ^{CG}		AF052938
Family Telestidae			
<i>Telestula</i> sp.	Kaena Point, Oahu, HI, 558 m	OAS-28	AF052914
Family Tubiporidae			
<i>Tubipora musica</i>	Bikini Atoll, Marshall Islands ^{NMNH}	79459	AF052909
Family Coelogorgiidae			
<i>Coelogorgia palmosa</i> (partial)	Aldabra, Seychelles, 60-80 ft ^{NMNH}	75637	AF052932
Suborder Alcyoniina			
Family Alcyoniidae			
<i>Acyonium gracillimum</i>	Genbank		Z92902
<i>Anthomastus</i> sp.	Keanapapa Pt., Lanai, HI, 1226 m	LAD-64	AF052881
<i>Bellonella rigida</i>	Genbank		Z49195
<i>Protodendron</i> sp.	Fieberling Guyot, 640 m	AD2321-8	AF052921
Family Nephtheidae			
<i>Lemnalia</i> sp.	Gulf of Eilat, Red Sea ^{TG}		AF052924
Family Nidaliidae			
<i>Siphonogorgia</i> sp.	Angulpelu Island, Palau, 110 ft ^{NMNH}	58584	AF052927
Family Xenidae			
<i>Xenia</i> sp.	Gulf of Eilat, Red Sea ^{TG}		AF052931
Suborder Scleraxonia			
Family Briareidae			
<i>Briareum asbestinum</i>	San Blas Islands, Panama ^{TG}		AF052912
Family Anthothelidae			
<i>Anthothela nuttingi</i>	Cross Seamount, HI, 1010 m	CR106-1	AF052922
<i>Erythropodium caribaeorum</i>	San Blas Islands, Panama ^{TG}		AF052915
Family Paragorgiidae			
<i>Paragorgia</i> sp.	Fieberling Guyot, 490 m	AD2301-1	AF052917
Family Coralliidae			
<i>Corallium kishinouyei</i>	Cross Seamount, HI, 1145 m	CR105-1, NMNH 94462	AF052918
<i>Corallium</i> cf. <i>ducale</i>	Cross Seamount, HI, 1370 m	CR202-2	AF052919

Family Melithaeidae			
<i>Melitella</i> sp.	Okinawa Archipelago, Japan, 16 m ^{AJ}	OK1078KM	AF052929
Suborder Holaxonia			
Family Acanthogorgiidae			
<i>Calcigorgia spiculifera</i>	Aleutian Islands, 431 m ^{NMNH}	not. cat.	AF052925
<i>Acanthogorgia</i> sp.	Bishop Seamount, HI, 1295 m	BI104-3	AF052907
Family Plexauridae			
<i>Eunicea laciniata</i>	Antigua, Caribbean Sea, 30 ft ^{NMNH}	73614	AF052926
<i>Calcigorgia granulosa</i>	Genbank		Z92900
<i>Euplexaura crassa</i>	Genbank		Z92901
Paramuriceid sp.	Cross Seamount, HI, 1145 m	CR105-5	AF052920
Family Gorgoniidae			
<i>Lophogorgia chilensis</i>	Santa Catalina Island, CA, ~15 m		AF052928
Family Ellisellidae			
<i>Juncella</i> sp.	Guam ^{TG}	92412	AF052936
<i>Juncella racemosa</i>	Okinawa, Japan, 45 m ^{NMNH}		AF052937
Family Ifalukellidae			
<i>Plumigorgia hydroides</i> (partial)	S. Satawan, Micronesia Chuuk, 30 m ^{AJ}	OCDN02224	AF052930
Family Chrysogorgiidae			
<i>Chrysogorgia chryseis</i>	Cross Seamount, HI, 1010 m	CR106-2	AF052913
Family Dendrobrachiidae			
<i>Dendrobrachia paucispina</i>	Great Australian Bight, 884-859 m ^{NMNH}	87768	AF052903
Family Primnoidae			
<i>Narella nuttingi</i>	Cross Seamount, HI, 1350 m	CR203-1	AF052881
<i>Narella bowersi</i>	Pensacola Seamount, HI, 1350 m	PN105-3	AF052905
Family Isididae			
<i>Lepidisis</i> sp.	Pensacola Seamount, HI, 1425 m	PN104-1	AF052906
<i>Orstomis crosnieri</i>	New Caledonia, 600 m ^{NMNH}	84774	AF052916

Order Pennatulacea			
Suborder Sessiliflorae			
Family Anthoptilidae			
<i>Anthoptilum</i> cf. <i>grandiflorum</i>	Keanapapa Pt., Lanai, HI, 1226 m	LAD-35	AF052933
Family Protoptilidae			
<i>Protoptilum</i> sp.	Bishop Seamount, HI, 1440 m	BI101-3	AF052911
Family Renillidae			
<i>Renilla reniformis</i>	Mission Bay, CA, 8 m ^{CG}		AF052581
Family Umbellulidae			
<i>Umbellula</i> sp.	Bahamas, 1447 m ^{NMNH}	54597	AF052904
Suborder Subselliflorae			
Family Pennatulidae			
<i>Leiopitilus fimbriatus</i>	Genbank		Z92903
Family Virgulariidae			
<i>Stylaula</i> sp.	Mission Bay, CA, 8 m ^{CG}		AF052934
<i>Virgularia</i> sp.	Mission Bay, CA, 8 m ^{CG}		AF052935
<i>Acanthoptilum</i> sp.	Mission Bay, CA, 8 m ^{CG}		AF052910

Dendrobrachia paucispina were acquired from Dr. Frederick Bayer of the National Museum of Natural History and Ardis Johnston of the Harvard Museum for Comparative Zoology. These specimens had been stored in ethanol for periods ranging from two to 50 years. No information was available as to whether the samples were fixed originally in ethanol or in formalin. Fresh specimens of octocorals and Actiniaria were collected by submersible from Hawaiian seamounts (using the Pisces V submersible, operated by the Hawaiian Undersea Research Laboratory at the University of Hawaii) and Fieberling Guyot (using the Alvin submersible, operated by the Woods Hole Oceanographic Institution). The specimens collected from these dives were frozen in liquid nitrogen or frozen at -20°C. Other octocoral specimens were collected in US waters by snorkeling or SCUBA. DNA extractions of several octocorals were donated by Dr. Tamar Goulet of the State University of New York, Buffalo.

DNA Extraction Protocol

The extraction protocols were based on those described by Coffroth et al. (1992) and Winnepeninckx et al. (1993). Five to ten polyps of fresh or frozen tissues were minced with a razor blade and placed in a 1.5-ml eppendorf tube with 600 µl of 2X cetyltrimethylammonium bromide (CTAB) buffer ((1.4M NaCl, 0.02M EDTA, 0.1M Tris-HCl (pH 8.0), 2% CTAB (Sigma Chemical Co.), and 0.2% beta-mercaptoethanol)). A plastic dounce was employed to further shear the tissue, and an additional 300 µl of 2X CTAB was added. The samples were placed at 55°C and digested with 5 µl of proteinase K (20 mg/ml) for approximately two hours. The tissues were extracted once with an equal volume of 24:1 chloroform:isoamyl alcohol, and precipitated in two volumes of cold 95% ethanol at -20°C overnight. The tubes were centrifuged at 10,000xg for 30 minutes, and the ethanol was removed. The pellets were washed in 500 µl cold 70% ethanol, and the tubes were centrifuged at 7,000xg for 15 minutes. The ethanol was removed, and the

pellets dried at room temperature. The pellets were resuspended in 50 µl of TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)) and placed at 4°C for three to four hours before visualization on an agarose gel.

The DNA extraction protocol for the archival specimens differed slightly. Five to ten polyps of the ethanol-stored specimens were placed on ice in 2 to 10 ml 2X CTAB buffer for up to 24 hours, with the buffer replaced several times during this period. The buffer was removed, and the tissue minced finely with a razor blade as above. The tissues were incubated with proteinase K at 55°C for 24 hours, with periodic agitation. Another 5 µl of proteinase K was added, and the tissues continued to digest for an additional eight to twelve hours. The subsequent extraction procedure followed as above.

DNA Amplification Protocols

Pipet tips with a filter barrier were used throughout this process to guard against contamination of the reactions. Negative controls were included during the DNA extractions and PCR reactions to detect contamination if it did occur. Each extracted DNA sample was diluted 1:10 in TE buffer, and 2 µl of that dilution was used in a 50-µl PCR reaction. Modified versions of the universal eukaryotic primers A and B (with the polylinkers removed) from Medlin et al. (1988) were used in the initial DNA amplifications of the 18S rRNA gene. Primer sequences are as follows: A (forward) 5'-AACCTGGTTGATCCTGCCAGT-3', B (reverse)-- 5'-TGATCCTTCTGCAGGTTACCTAC-3'. I found the 18S rRNA gene to be roughly 1800 basepairs (bp) in length in anthozoans. Thirty-five cycles of PCR were carried out using a Perkin Elmer Thermal Cycler 480. The DNA was denatured at 94°C for 45 seconds, the primers and template were annealed at 55°C for one minute, and the original DNA strand was extended at 72°C for 90 seconds. These 35 cycles were followed by a

five minute extension at 72°C. The product was visualized on a 1% agarose gel, and then prepared for TA-cloning.

For those archival specimens that yielded no visible PCR product, a second PCR reaction was conducted using 1 µl of product from the initial PCR reaction as the template. The amplified negative control (no DNA was added to the tube) from the initial PCR reaction was included in the second reaction, using 1 µl of the original negative control as template. The primers used for the second PCR reaction were chosen to insure that at least one primer annealed internally to the initial A and B primers. The internal primers were selected from a combination of universal eukaryotic primers and a set of octocoral-specific primers that I designed. The universal primers are the following: 373 (forward) 5-'GATTCCGGAGAGGGAGCCT-3' and 1200 (reverse) 5'-GGGCATCACAGACCTG-3' (Weekers et al. 1994), 514 (forward) 5'-GTGCCAGCMGCCGCGG-3', 1055 (forward) 5'-GGTGGTGCATGGCCG-3', and 1055 (reverse) 5'-CGGCCATGCACCACC-3' (Elwood et al. 1985), and 536 (reverse) 5'-WATTACCGCGGCKGCTG-3' (Lane et al. 1985). The taxon-specific primers were designed to amplify anthozoan DNA, but not DNA from the potential contaminants. The octocoral-specific primers were designed from alignments of GenBank sequences of actinarians (listed in Table 1), two fungi (*Cryptococcus neoformans*, GenBank Accession #L05428, and *Bullera unica*, #D78330), potential epibionts from Mollusca and Crustacea (*Mytilus galloprovincialis*, #L33451, and *Stenocypris major*, #Z22850), and a zooxanthella symbiont (*Symbiodinium* sp., #M88509). Octocoral sequences derived from frozen tissue in this lab (Table 1) were verified as cnidarian through a BLAST search of GenBank and were also used in the primer design. Octocoral-specific primer sequences are the following: 705 (forward) 5'-GGTCAGCCGTAAGGTTT-3', 705 (reverse) 5'-CATACCTTTCGGCTGACC-3', 900 (forward) 5'-GTTGGTTTTTTGAACCGAAG-3', 900 (reverse) 5'-CTTCGGTTCTAGAAACCAAC-3', 1560 (reverse) 5'-GGTGAAGGAGTTACTCGATG-

3'. PCR primer pairs were chosen to include at least one octocoral-specific primer, and to amplify the largest fragment possible from the archival specimens. Further details and the rationale behind this technique are described elsewhere (Berntson & France in prep).

Determination of DNA Sequences

The final PCR product was cloned using the Original TA Cloning Kit (Invitrogen Corporation). The PCR product was ligated into the pCR 2.1 cloning vector, then transformed into a strain of INV α F' cells. The plasmid was isolated using the Wizard Miniprep DNA Purification Kit (Promega Corporation) and subsequently used as a template for cycle sequencing reactions, using the SequiTherm EXCEL Long-Read DNA Sequencing Kit-LC (Epicentre Technologies). DNA sequences were determined for both the forward and reverse strands of the gene. The reactions were run on a LI-COR 4000 DNA Sequencer, using infrared-labeled primers: M13 (forward) 5'-CACGACGTTGTAAACGAC-3', M13 (reverse) 5'-GAATAACAATTTACACAGG-3', 514 (forward) 5'-TCTGGTGCCAGCASC CGCGG-3', 536 (reverse) 5'-TGGWATTACCGCGGSTGCTG-3', 1055 (forward) 5'-GTGGTGGTGCATGGCCG-3', 1055 (reverse) 5'-AAGAACGGCCATGCACCAC-3'. The resulting images were interpreted using the BioImage gel reader program.

Sequence Analysis

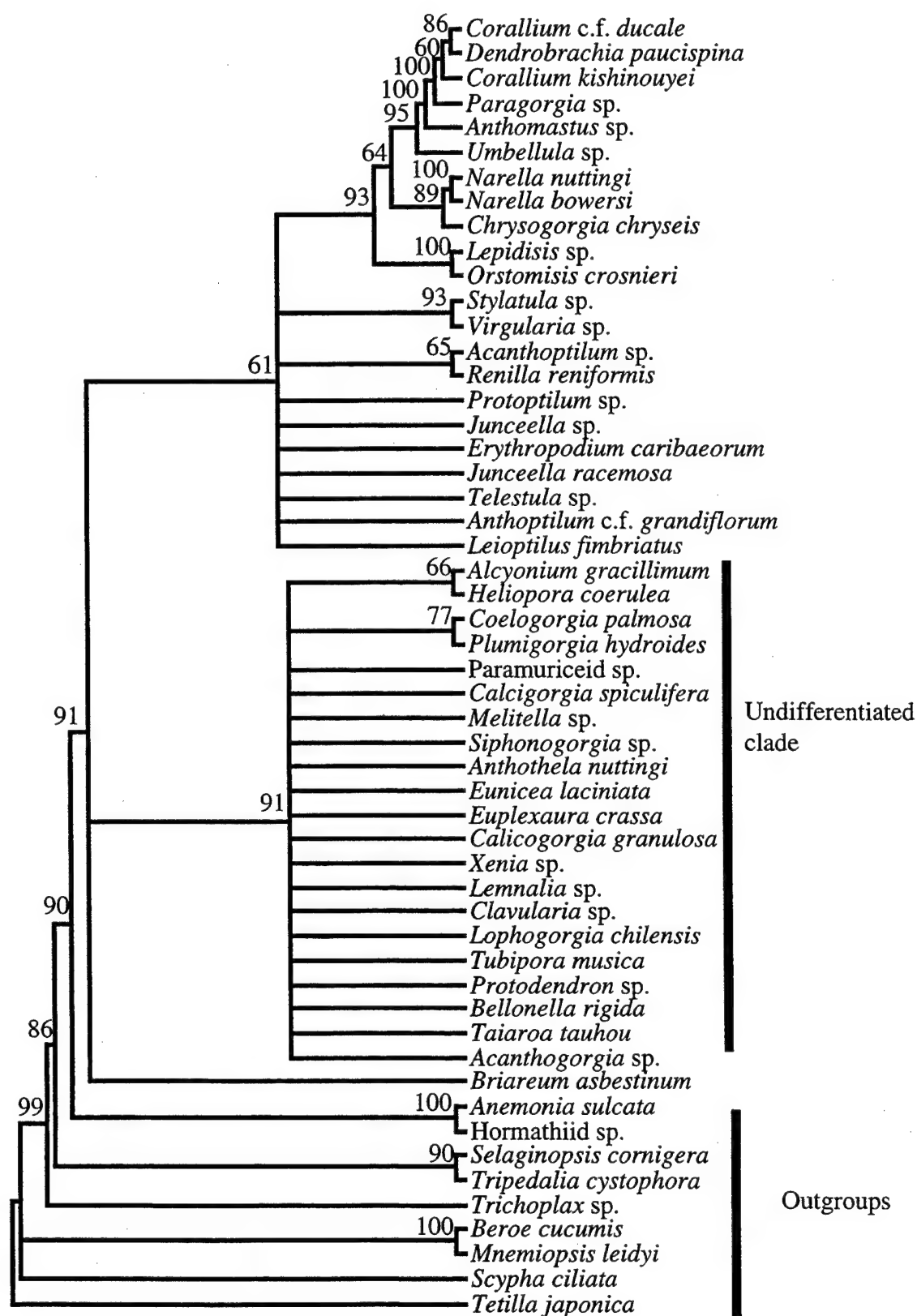
DNA sequences were aligned first by eye, taking into account secondary structure models, and with the alignment program Clustal W 1.6 (Thompson et al. 1994). Regions of uncertain alignment were eliminated from the final analyses (the nexus file is available upon request). In total, 1640 basepairs were used in the analyses, of which 413 out of 689 variable sites were parsimony-informative, and 276 were parsimony-uninformative.

All analyses were performed using test versions of PAUP* (versions 4d61 and 4d63) written by David Swofford (Swofford 1996, betatest version). An initial parsimony analysis of the entire dataset was performed, which resulted in 35,000 equally parsimonious trees. The likelihood scores were calculated for one of the most parsimonious trees, using a variety of evolutionary models. The purpose for this procedure was to identify the simplest model of evolution that was still accurate for this data set; the simpler model will have a lower variance (Rzhetsky & Nei 1995) and will be less computationally intensive. Using the Likelihood Ratio Test (LRT) as described by Huelsenbeck and Rannala (1997), the likelihood scores were calculated for each ML model and compared to the most complex model, which is a general time-reversible (GTR) model incorporating unequal base frequencies and among-site substitution heterogeneity. The values of the LRT are approximately chi-square distributed, with the degrees of freedom equal to the difference in free parameters between the models being tested. Models for which the LRT score was greater than the Chi-square critical value were rejected as not being sufficiently accurate.

A strict consensus tree from the initial parsimony search was calculated, and it indicated a large undifferentiated clade (data not shown). A bootstrap analysis using ML-based distance calculations was also performed, and the results showed strong support (bootstrap value = 91) for the existence of the same undifferentiated clade (Fig. 2). The topology of the taxa within this clade was fixed for subsequent analyses, based on one of the equally parsimonious trees found. This was done for computational reasons, as it effectively reduced the number of taxa in the analysis from 53 to 32. Without fixing this clade, much of the computational time would have been utilized by these taxa, for which there was very low phylogenetic signal.

The outgroups chosen for these analyses were two species from the Phylum Porifera, two species from the Phylum Ctenophora, one species from the Phylum

Fig. 2 **Bootstrap analysis using distances calculated with maximum likelihood parameters, showing the undifferentiated clade. Undifferentiated clade and outgroups identified by bars. Numbers at nodes are the percent that branch occurred in subsampled replicates. No taxa were fixed for this computation. Horizontal branch length does not reflect genetic distance among taxa.**



Placozoa, four other species from the Phylum Cnidaria (one each from the classes Hydrozoa and Cubozoa, and two species of anemones, Class Anthozoa) (Table 1). These species were previously shown to be appropriate outgroups for phylogenetic analyses of the subclass divisions within the Anthozoa (Berntson et al. in prep). Five replicates of a ML analysis were performed using random addition of taxa to find the most likely trees given this data set. Trees were viewed using PAUP* and the free-ware program TreeView (Page 1996).

Likelihood scores were also used to test specific phylogenetic hypotheses relating to the questions addressed with this study. Alternative evolutionary hypotheses were formed by manipulating tree topologies using the computer program MacClade (Maddison & Maddison 1992). The Kishino-Hasegawa test (KH Test) (Kishino & Hasegawa 1989) within the PAUP* program was used to compare the likelihood scores of those topologies.

I compared genetic divergences within the Octocorallia to those found in the Hexacorallia, as well as to divergences measured in other invertebrates using 18S ribosomal sequences. I used PAUP* to calculate the basic percent divergence values (*p*-distance) and the ML-corrected distances, using the aligned and edited dataset. The divergences for the Hexacorallia were calculated from the data set from another study (Berntson et al. in prep).

Results

The results from the Likelihood Ratio Test (LRT) of the original parsimony tree showed statistically ($P < 0.05$) that simple parsimony and distance models were insufficient to represent the evolution of this gene. The only appropriate model for the analysis of this data set was a General Time-Reversible (GTR) model with unequal base frequencies and among-site heterogeneity. All simpler models were statistically inferior to the full GTR model for explaining this tree, given these data. The parameters estimated from the

parsimony tree (Table 2) were used in a heuristic ML search consisting of five replicates, with random addition of sequences. The parameters were re-estimated using the most likely tree that was produced from that ML analysis, to insure there were no significant changes. The most likely trees were found within the first replicate in all analyses conducted.

Three equally likely trees were produced from the ML analysis, and a representative of these trees is shown (Fig. 3). Three distinct clades were evident within the Octocorallia, none of which corresponded to current taxonomy. The most likely trees differed in the branching order of the basal nodes separating the three clades. This branching order could not be resolved statistically with the present sequence information.

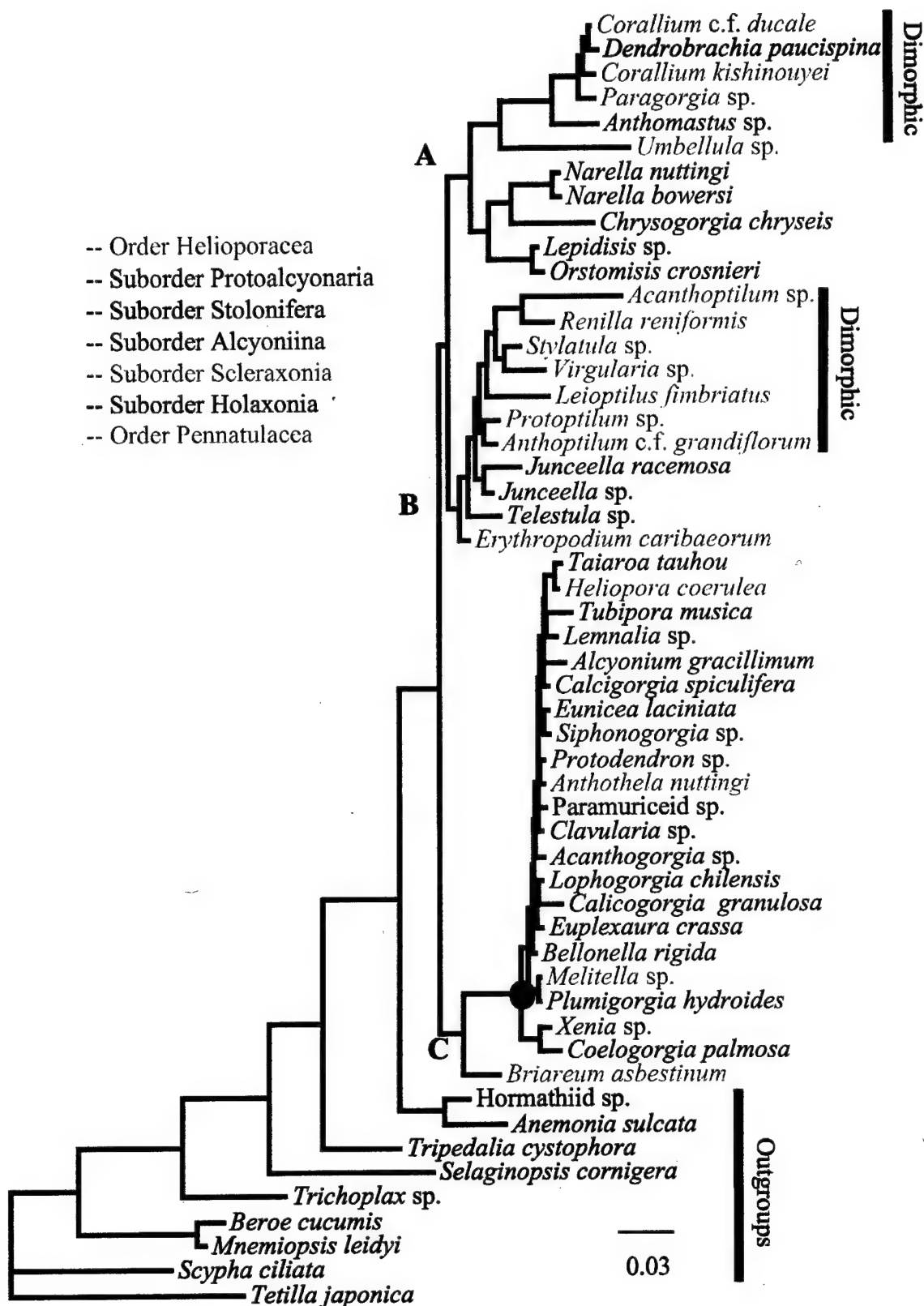
Members of most suborders were represented in all three clades. Clade A (Fig. 3) had the most phylogenetic structure within it, as indicated by bootstrap values associated with its topology (Fig. 2). Clade A (Fig. 3) contains primarily holaxonians (families Chrysogorgiidae, Isididae, and Primnoidae), but also members from the Scleraxonia, Pennatulacea, and Alcyoniina. Clade B contains the majority of the Pennatulacea, as well as members of the Stolonifera, Scleraxonia, and Holaxonia. Clade C is essentially undifferentiated, and contains members of all major groups except Pennatulacea. There is very little phylogenetic signal present within this clade.

The most likely trees provided support for the current taxonomy at the level of Family, for some of the families represented by multiple species. The families Ellisellidae, Isididae, and Primnoidae (Order Alcyonacea: Suborder Holaxonia) constituted monophyletic groups based on the species chosen here. The most likely tree showed the Coralliidae (Suborder Scleraxonia) to be paraphyletic, with the inclusion of the genus *Dendrobrachia*. *Dendrobrachia* could be placed as sister-taxon to the two species of *Corallium* without reducing the likelihood of the tree significantly (KH Test, $P < 0.05$). The families Alcyoniidae (Order Alcyonacea: Suborder Alcyoniina), Anthothelidae (Order

Sequence Parameter	Value
Base frequencies	
A	0.258
C	0.206
G	0.264
T	0.272
Substitution R matrix	
A/C	1.0257
A/G	2.6331
A/T	0.9627
C/G	1.3927
C/T	4.4228
Proportion of invariant sites	0.3761
Gamma distribution	
α	0.6455

Table 2 Sequence parameter values calculated from the 18S rDNA used in the present study. Values used as input for maximum likelihood analyses. The R matrix contains base-specific substitution rates. The gamma distribution parameter α is the inverse of the coefficient of variation of the substitution rate.

Fig. 3 One of three equally likely trees produced from maximum likelihood analyses, with the topology of species in clade C (circled) fixed ($-\ln$ likelihood = 11129.976). The relative positions of nodes A, B and C are interchangeable and equally likely. Clades marked with vertical bars contain dimorphic species. Horizontal branch length reflects genetic distance among taxa. Colors represent the major taxonomic groupings within the Octocorallia. The seven species in black at the base of the tree are the outgroups.



Alcyonacea: Suborder Scleraxonia), and Virgulariidae (Order Pennatulacea) did not appear to be monophyletic. The associations within the families Acanthogorgiidae and Plexauridae (Order Alcyonacea: Suborder Holaxonia) could not be determined from these data, as they fell within the undifferentiated clade C.

The morphologically well-defined groups within the Octocorallia were not well supported genetically. The majority of the species within the Order Pennatulacea branched with Clade B, but the genus *Umbellula* fell in Clade A, basal to *Anthomastus*. *Umbellula* could not be moved without reducing the likelihood of the tree significantly (KH Test, $P < 0.05$). The remaining Pennatulacea formed a monophyletic clade, at the base of which fell species from three different suborders. The sole representative of the other well-defined group of octocorals, the Heliporacea, could not be distinguished from the undifferentiated Clade B. Its placement, however, may be an artifact resulting from an incomplete sequence (541 bp out of 1646 were used). The clear association of the 'restricted Holaxonia' was also not supported in these trees, as the four families were spread throughout all three clades.

Four taxa (*Erythropodium*, *Telestula*, and two species of *Junceella*) were found at the base of the pennatulacean clade in each of the three most likely trees (Fig. 3). The distinct morphology of the Pennatulacea does not predict a close association with these taxa. I tested the support for the placement of these four taxa with the Pennatulacea by constraining them into each of clades A and C, and performing an heuristic search for the most likely tree given each of the constraints. These four taxa could be placed at the base of either Clade A or Clade C without reducing the likelihood of the tree significantly (KH Test, $P < 0.05$).

Dendrobrachia paucispina was closely affiliated with the genera *Corallium* and *Paragorgia*, with 100% bootstrap support (Fig. 2). *Dendrobrachia* could be moved to all

positions on the tree near *Paragorgia* and the two species of *Corallium* without reducing the likelihood of the tree significantly (KH Test, $P < 0.05$).

The morphological character of dimorphism was found in two separate groups: the Pennatulacea, and the group containing *Umbellula*, *Anthomastus*, *Paragorgia*, and *Corallium* (Fig. 3). I forced monophyly of the dimorphic species to investigate the possibility that this character arose once within the Octocorallia. The resulting topology (Fig. 4) did not decrease the likelihood significantly from the best trees (KH Test, $P < 0.05$). *Erythropodium*, *Telestula*, and the two species of *Junceella* in the previous paragraph could be moved to any of positions 1, 2, or 3 on this tree without reducing the likelihood significantly (KH Test, $P < 0.05$).

The genetic divergences measured using p - and ML-distance calculations from these 18S sequence data showed similarities between the Hexacorallia and the Octocorallia, as well as with other invertebrates at the higher taxonomic levels (between classes within Cnidaria and orders within the Hexacorallia and Octocorallia) and at the lower levels (within genera). Divergences at the intermediate taxonomic levels (between suborders and within families) did not agree as closely between the Anthozoa and other invertebrates (Table 3). The ML-corrected distances were slightly greater than the p -distance values. The p -distance divergence between classes in the Phylum Cnidaria was approximately 6.8-15%, which is comparable to those values found among classes of echinoderms (6.0-12.2%) (Wada & Satoh 1994). The divergence among the orders within the hexacorals (1.5-8.1%) was similar to the divergence found between the octocorallian orders Pennatulacea and Alcyonacea (1.3-9.2%), and between the Helioporacea and the Alcyonacea (0.34-8.15%). The divergence within genera was approximately equivalent for hexacorals (0.5-1.07%) and octocorals (0.6-1.6%), although it was greater than that found in the bivalve mollusk *Mytilus* (0.1-0.6%) (Kenchington et al. 1995).

Fig. 4 The resulting likelihood tree when the dimorphic species are constrained to a single clade ($-\text{Ln likelihood} = 11153.843$). The topology of this tree is not significantly less likely than the most likely tree (KH Test, $P < 0.05$). The species *Telestula* sp., *Erythropodium caribaeorum*, *Junceella racemosa* and *Junceella* sp. can be placed at positions 1, 2 or 3 without a significant reduction in the likelihood of the tree (KH Test, $P < 0.05$). The seven species in black at the base of the tree are the outgroups.

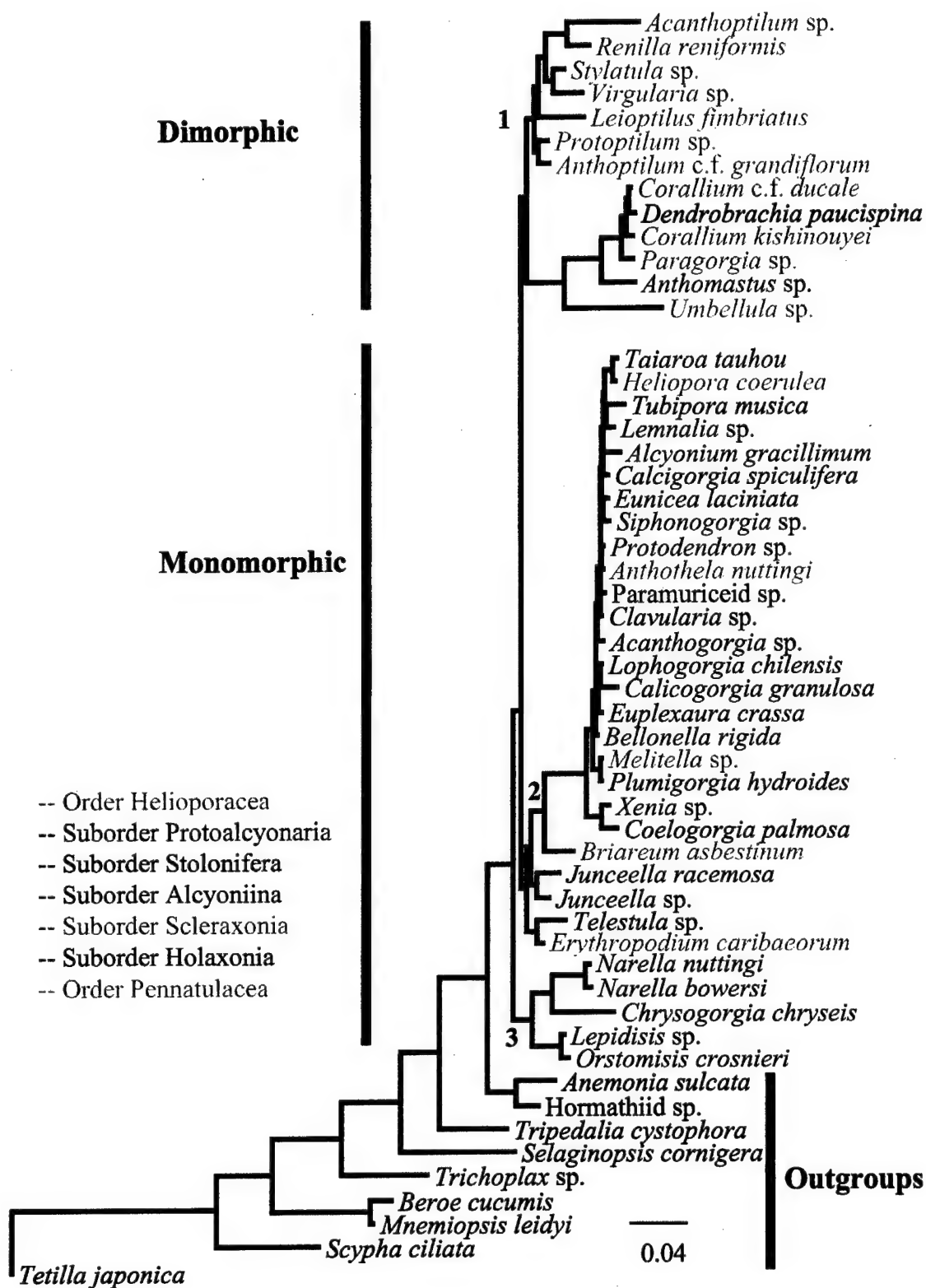


Table 3 **Genetic divergences measured from nuclear 18S rDNA.**
Values represent raw *p*-distances and maximum likelihood-
corrected distances. Estimates were made from the aligned,
edited data set.

Category	Taxa	<i>P</i> -distance (%)	ML distance (%)	Reference
Between classes	Cnidaria, without Ceriantharia Cnidaria, with Ceriantharia Echinodermata	6.8-15.0 6.8-15.2 6.0-12.2	8.5-21.8 8.5-25.3 N/A	present study present study Wada and Satoh 1994
Between subclasses	Anthozoa, without Ceriantharia Anthozoa, with Ceriantharia	4.2-10.7 4.2-13.8	5.1-14.9 5.1-21.2	present study present study
Between orders	Hexacorallia Pennatulacea/Alcyonacea Helioporacea/Alcyonacea	1.5-8.1 1.3-9.2 0.3-8.2	1.6-10.2 1.4-12.8 0.3-11.6	present study present study present study
Between suborders	Hexacorallia Pennatulacea, without <i>Umbellula</i> Pennatulacea, with <i>Umbellula</i>	0.4-4.5 1.6-5.1 1.6-9.2	0.4-5.1 1.6-5.8 1.6-12.0	present study present study present study
Within families	Decapoda Hexacorallia Octocorallia	11.0-12.9 0.4-2.6 0.4-7.6	N/A 0.6-2.9 0.4-9.3	Kim and Abele 1990 present study present study
Within genera	Mytilidae Hexacorallia Octocorallia <i>Mytilus</i>	2.7-4.5 0.5-1.1 0.6-1.6 0.1-0.6	N/A 0.5-1.1 0.6-1.7 N/A	Kennington et al. 1995 present study present study Kennington et al. 1995

N/A: Not available

The divergence between suborders within the Hexacorallia (0.4-4.5%) was similar to the divergence among suborders within the Pennatulacea (1.6-5.1%), exclusive of *Umbellula* sp. which did not branch with the other Pennatulacea. The inclusion of *Umbellula* raised the upper bound of divergence from 5.1% to 9.2% between suborders. This level of divergence was much lower than that found between suborders of decapod crustaceans (11-12.9%) (Kim & Abele 1990). The genetic divergence among the remaining suborders was more difficult to interpret for the Octocorallia, because the members of many suborders were mixed within each clade in my most likely trees (Fig. 3). Therefore I measured the divergence between and within the three major clades from our ML analyses. The divergence between the octocorallian clades ranged from 2.5-9.9%. Within clades, the divergence ranged from 0.4-8.4% in Clade A, 0.9-6.5% in Clade B, and 0.0-5.8% in Clade C. The divergence in Clade A was roughly equivalent to the ordinal-level divergence within the Hexacorallia, but the divergence within clades B and C was closer to the subordinal-level divergence within the hexacorals and Pennatulacea. The within-family divergence of the octocorals (0.4-7.6%) was much higher than that within the hexacorals (0.4-2.6%) as well as those found in Mytilidae (2.7-4.5%) (Kenchington et al. 1995).

Discussion

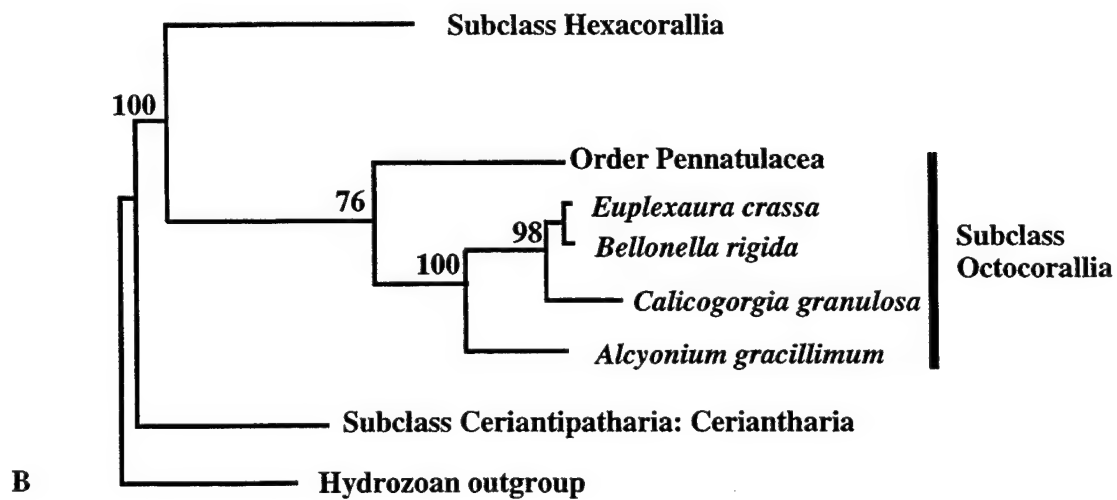
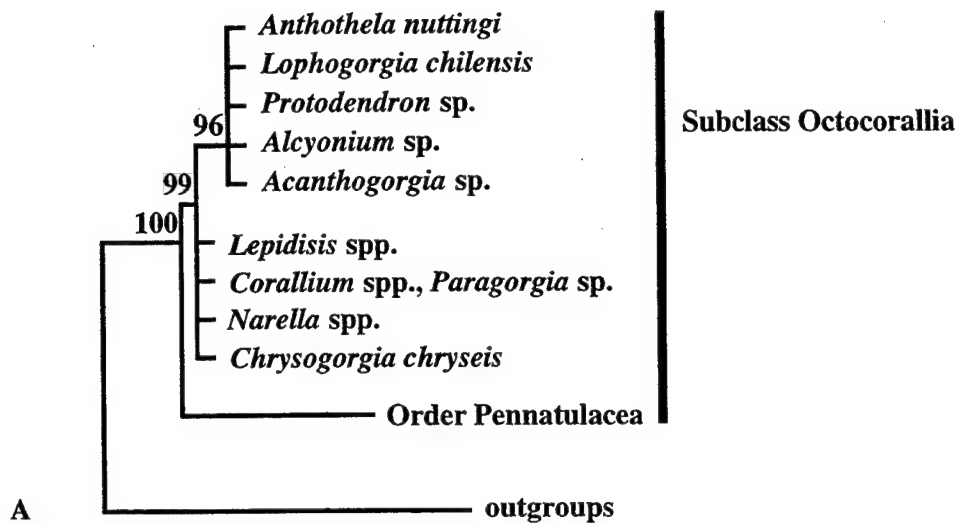
Phylogenetic analyses of nuclear 18S sequences from Octocorallia do not support either the historical or the current classification system. The phylogenetic clades evident within the Subclass Octocorallia did not correspond to the morphologically defined orders or suborders (Fig. 3). The clades present were similar, however, to the associations found by France et al. (1996) using partial mt 16S rDNA (Fig. 5A). Mitochondrial 16S sequence information also distinguished three groups, with a pennatulacean as the basal branch (corresponding to my Clade B), an undifferentiated clade containing species found in my

Fig. 5

Previous molecular phylogenetic studies examining relationships within the Octocorallia. Numbers at nodes represent bootstrap values.

A) From France et al. (1996), based on mitochondrial 16S rDNA

B) From Song and Won (1997), based on nuclear 18S rDNA



Clade C, and a further unresolved clade of some of the same species found in my Clade A. A previous study using partial 18S rRNA sequences (Song & Won 1997) also found the one pennatulacean species branching first within the Octocorallia, but only had four other species representing the remaining octocorals (Fig. 5B). All four of those species corresponded to families within my Clade C.

The morphologically well-defined groups within the Octocorallia (Pennatulacea and Helioporacea) did not form monophyletic clades based on 18S sequence information. The pennatulacean species in this analysis formed a monophyletic group, with the exception of the genus *Umbellula* which fell in Clade A. Four non-pennatulacean species branched at the base of the pennatulacean clade. I could not verify the putative ancestral position of the Pennatulacea within the Octocorallia because the branching order of the three clades could not be established with these data. The helioporacean species *Heliopora* was found in Clade C and was not separate from the other octocorals, but this may be a result of using an incomplete sequence in the analyses. The 'restricted Holaxonia,' the final group of species considered by Bayer (1981) to be distinct, also failed to form a unified phylogenetic group. Members of those four families (Ifalukellidae, Chrysogorgiidae, Primnoidae, and Ellisellidae) could be found in all three clades.

Morphological taxonomy does not predict that the genus *Umbellula* would be more closely allied with *Anthomastus*, *Paragorgia*, and *Corallium* rather than the other Pennatulacea, given the characteristic morphology of the pennatulaceans. *Umbellula* is unique among the Pennatulacea, however, in that all of its autozooids form a cluster at the end of its rachis (Hickson 1930). Morphologically, *Umbellula* does have similarities to the alcyonacean species *Anthomastus* with which it is associated. *Anthomastus* is dimorphic, as are *Corallium* and *Paragorgia* (also within that subclade). *Anthomastus* possesses several large autozooids and many small siphonozooids, forming a distinct body at the top of a stalk devoid of polyps (Bayer 1993a). Both *Umbellula* and *Anthomastus*

have very large polyps. This resemblance is primarily superficial, however, and further investigation concerning morphological similarities between *Umbellula* and *Anthomastus* are necessary.

The unusual genus *Dendrobrachia* was clearly associated with the species of *Corallium* and *Paragorgia* included in these analyses. Opresko and Bayer (1991) concluded that *Dendrobrachia* was more closely related to the Family Chrysogorgiidae, based on its resemblance to the two chrysogorgiid species *Trichogorgia lyra* and *Malacogorgia* (= *Trichogorgia*) *capensis*. *Dendrobrachia* has a solid, chitinous axis devoid of calcium, and no free sclerites. *Malacogorgia capensis* has an identical, chitinous axis, and the axis of *Trichogorgia lyra* is similar but contains calcium deposits. Both *Trichogorgia lyra* and *Malacogorgia capensis* lack free sclerites, making them the only species of Scleraxonia or Holaxonia for which this is true (Opresko & Bayer 1991). The representative of the Family Chrysogorgiidae in this analysis (*Chrysogorgia chryseis*) branched within Clade A, as did *Dendrobrachia*, but *Dendrobrachia* could not be forced to branch with *Chrysogorgia* or its sister taxa without reducing the likelihood of the tree significantly (KH Test, $P > 0.05$).

The morphological similarities of *Dendrobrachia* to *Corallium* and *Paragorgia* are not clear. Although the axis of *Corallium* is solid, as in *Dendrobrachia*, it is entirely calcareous (Bayer 1964). The axis of *Paragorgia* contains chitin as well as calcium, but its central core is hollow (Bayer 1993b). There are no clear morphological connections between *Dendrobrachia* and *Corallium*.

There is also morphological evidence for the association of the genera *Anthomastus* and *Paragorgia* as found from 18S data (Fig. 3). Broch and Horridge (1957) proposed uniting the two genera within the Family Paragorgiidae. Both genera are dimorphic, and they share similar forms of sclerites. Broch and Horridge asserted that *Anthomastus* differed from *Paragorgia* only in the lack of chitin in its central axis, a character they felt to

be less important than the similarity in polyp and sclerite morphology. Bayer (1993a) dismissed the importance of sclerite similarity, however, noting that the particular form of sclerite the two genera share is fairly common among alcyonaceans.

The morphological similarities between *Corallium* and *Paragorgia* are numerous. *Paragorgia* was historically considered to be in the order Alcyonacea (now the Suborder Alcyoniina), but was later placed in the Suborder Scleraxonia (Bayer 1973). Their axial structures differ slightly. The axis of *Corallium* is solid and exclusively calcareous, whereas the axis of *Paragorgia* is chambered and contains both calcium and chitin. The growth form and sclerite structure of *Corallium* and *Paragorgia*, however, are virtually identical. *Corallium* and *Paragorgia* are the only genera within the Suborder Scleraxonia exhibiting colony dimorphism (Bayer 1964). The close phylogenetic association of the two genera is also indicated from mt16S rDNA analyses (Fig. 5) (France et al. 1996).

The importance of skeletal structures has been implicit in the creation of the current morphological taxonomy, specifically the subordinal divisions (historically ordinal divisions). The appearance of members of all suborders in most of the clades in my trees suggests that skeletal structure may not be the primary character indicative of evolutionary patterns.

One character that did correspond roughly to the observed branching pattern was the presence or absence of dimorphism in colony morphology. Dimorphism occurs in all species of the Pennatulacea, as well as several species in the Family Alcyoniidae (Suborder Alcyoniina), and the two families Coralliidae and Paragorgiidae (Suborder Scleraxonia). The members of the Suborder Holaxonia are exclusively monomorphic, as are the members of the Protoalcyonacea and the Stolonifera. The Order Helioporacea is also monomorphic (Bayer 1973). There were two small clades of dimorphic species from my data: Clade B contained the majority of the Pennatulacea, and half of Clade A contained the remaining pennatulacean and the other dimorphic species in this analysis (Fig. 3). The taxa at the

base of the pennatulacean clade (B) were monomorphic, but could be moved to clades A or C without reducing the likelihood of the tree significantly. All of the species included in our study from the Suborder Alcyoniina are monomorphic and fell in Clade C, with the exception of *Anthomastus* which is dimorphic and branched with the dimorphic species in Clade A. The correlation between the phylogenetic groupings I found within the Order Octocorallia and monomorphism vs. dimorphism is striking.

The morphological similarities among the dimorphic species are so few, however, that it would seem likely that dimorphism arose multiple times within the Octocorallia. Morphologically, it is difficult to imagine a single common ancestor for all of the dimorphic species. The monophyly of dimorphism can be forced topologically, however (Fig. 4), without resulting in a significantly lower likelihood for the overall tree (KH Test, $P < 0.05$). Dimorphism may be a functional constraint, however, based on colony structure rather than common ancestry. The branched, lobate, and massive octocorals are all dimorphic, which suggests that dimorphism arose in response to the need for transporting water more efficiently through a large structure (Bayer 1973). My analyses cannot reject either the monophyly or the polyphyly of the dimorphic character.

The one clear exception to the pattern of dimorphism found in our trees was the genus *Dendrobrachia*, which is entirely monomorphic, yet clustered tightly with the dimorphic genera *Corallium* and *Paragorgia*. The simplest way to explain the association is to assume the dimorphism trait was lost during *Dendrobrachia*'s evolution, although more evidence is needed to support such a conjecture.

The morphological characters used to derive the current classification system do not appear to be reflected in the genetic associations I have found. The relative importance of several of these characters to the evolutionary history of the octocorals needs to be re-examined. The nuclear 18S rDNA gene, however, may not be appropriate for these particular analyses. Although the 18S gene has been used to elucidate phylogenetic

relationships at the taxonomic levels of order and suborder in previous studies of other invertebrate taxa, the level of divergence 18S rDNA exhibited in the present study did not allow me to distinguish definitive phylogenetic relationships within the Subclass Octocorallia. Analyses of 18S rDNA sequences could not distinguish relationships within a large subset of species within this subclass, those species branching in Clade C of our analyses (Fig. 3). The high level of divergence found within families may reflect incorrect familial designations within the octocorals. Indeed, Bayer (Bayer 1956) comments that several genera are incorrectly included in existing families, and that these relationships need to be reassessed in many cases. There were distinct phylogenetic groups indicated by 18S sequence information, corresponding to the three clades we found, but relationships within those clades remains equivocal. Sequences from a different gene, exhibiting higher levels of divergence, may be necessary to delineate relationships beyond those found in this study.

Conclusions

This study has shown that the traditional, morphological taxonomy of the Subclass Octocorallia is not reflected in phylogenetic structure as indicated from 18S rRNA sequence information. Three phylogenetically distinct clades were evident, but they do not correspond to current subordinal divisions. Nuclear 18S rDNA sequence information did indicate some genetic structure within the Octocorallia, particularly in Clade A (Fig. 3). The largest clade (Clade C) contained nearly half of the species used in these analyses, but the phylogenetic signal was so low that the relationships within this clade could not be determined from these sequence data. A similar clade was also present in an earlier analysis using mt16S rDNA (France et al. 1996), which may indicate this is a ribosomal-specific pattern. More likely, however, the branching similarity resulting from the two independent data sets suggests that low levels of divergence are truly reflective of the

evolutionary history of these species. These low levels of divergence are suggestive of a rapid radiation in the evolutionary history of these taxa. The morphological continuum present within the Octocorallia and the lack of an extensive fossil record, however, make it very difficult to substantiate such an hypothesis using these characters.

The two morphologically well-established orders, the Pennatulacea and the Helioporacea, did not constitute entirely distinct genetic entities in these analyses. The majority of the pennatulaceans in this study were grouped together in a monophyletic clade, but the genus *Umbellula* was phylogenetically distinct from the other members of its order. The level of divergence between *Heliopora* and the remaining Octocorallia was similar to the divergence between the Pennatulacea and the Alcyonacea, but *Heliopora* clustered tightly within the undifferentiated Clade C (Table 3). This close association suggests that the Helioporacea are not highly differentiated from the Alcyonacea, although additional specimens and complete sequences from the Helioporacea may be necessary to confirm this hypothesis. Additional specimens from the Pennatulacea and *Umbellula* would be essential for confirming or refuting the genetic divergence of *Umbellula* from the otherwise monophyletic Pennatulacea.

The morphological characters of axial composition, sclerite form and arrangement, and general colony configuration are the primary characters that have been used to create the ordinal and subordinal groupings within the Subclass Octocorallia. There was very little correlation between those characters and the phylogenetic groups indicated with 18S sequence information. The character of monomorphism vs. dimorphism appeared relatively consistent in the trees I have produced, as all of the dimorphic species in this analysis clustered in two clades. The placement of the monomorphic genus *Dendrobrachia* was the one exception to the unity of the dimorphism character. *Dendrobrachia* branched very closely to the genera *Paragorgia* and *Corallium*, both of which are dimorphic. The axial morphology of *Dendrobrachia* and its lack of sclerites suggest a closer affinity with

the family Chrysogorgiidae, which was not supported from these analyses. Further morphological study and sequence information from additional genes will be necessary to clarify the phylogenetic relationship of *Dendrobrachia* to the other octocorals.

This study constitutes a broad-scale survey of genetic differentiation of nuclear 18S rDNA sequences across the entire subclass, representing the majority of the morphological diversity present in the subclass. The morphological characters that have been used to devise the traditional taxonomy of the octocorals do not correspond to the phylogenetic divisions reflected in genetic sequence information. The phylogenetic structure that was present indicated a well-supported clade primarily containing the dimorphic Scleraxonia, and a large undifferentiated clade containing half of the species from these analyses. The majority of the pennatulaceans formed a monophyletic group, with the exception of *Umbellula*. The remaining species fell into weakly-supported clades that could be moved without reducing the likelihood of the tree significantly. This branching pattern was upheld by analyses of mt 16S rDNA, which constitutes a second independent molecular character. The importance of the morphological characters used to create the traditional taxonomies may need to be reassessed as they relate to the evolution of these species. Additional characters will be required, either morphological characters or sequences from additional genomic regions, to further clarify the evolutionary progression of the Subclass Octocorallia.

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Chapter 5

Conclusions

Summary

The goal of this thesis project was to build a more complete molecular phylogeny of the Class Anthozoa than currently exists, based on the nuclear 18S rRNA gene. I addressed four primary questions concerning the phylogenetic divisions within the Anthozoa. In Chapter 3, I examined the subclass divisions within the Anthozoa, specifically to address the evolutionary relevance of the Subclass Ceriantipatharia. The orders Ceriantharia and Antipatharia were originally classified in the Subclass Hexacorallia, but were subsequently assigned to their own subclass. Previous molecular studies had suggested that the orders Ceriantharia and Antipatharia are genetically disparate, but the taxonomic sampling in those studies was insufficient to determine the precise affinities of the two orders relative to the remaining Anthozoa.

My analyses showed the orders within the Subclass Ceriantipatharia (Antipatharia and Ceriantharia) to be genetically dissimilar. Therefore, this subclass does not represent an evolutionarily relevant grouping. The Antipatharia were most closely related to the Zoanthidea (Subclass Hexacorallia), and the Ceriantharia appeared to be ancestral to the Hexacorallia. The exact phylogenetic position of the Ceriantharia, however, could not be determined with these sequence data.

I also examined the ordinal divisions within the Hexacorallia and the Ceriantipatharia in Chapter 3, to determine if the morphologically derived divisions are monophyletic or polyphyletic. The Order Scleractinia was monophyletic based on previous studies of both nuclear 18S rDNA and 28S rDNA, but polyphyletic based on mitochondrial 16S rDNA. The Order Actiniaria appeared to be monophyletic based on mt 16S rDNA, but was polyphyletic from the 18S and 28S studies. The 28S rDNA sequence information suggested the Order Corallimorpharia is also polyphyletic.

The Subclass Octocorallia and the hexacorallian orders Antipatharia, Zoanthidea and Actiniaria were each monophyletic based on my 18S sequence analyses. The Scleractinia and Corallimorpharia appeared to be polyphyletic and were closely associated with each other, but their exact relationship could not be determined reliably with these data. These data also could not be used to distinguish the branching hierarchy for the orders within the Hexacorallia.

Chapter 4 addressed the phylogenetic divisions within the Subclass Octocorallia. Traditional taxonomy was based on very few morphological characters, and the evolutionary significance of any given character is unclear. The traditional taxonomic system divided the subclass into seven orders. Recent taxonomic revisions retained two of the orders (Helioporacea and Pennatulacea) but combined the remaining species into a single order (Alcyonacea) with five subordinal divisions. Mitochondrial 16S rDNA suggested that neither the historical ordinal-level nor the current subordinal-level divisions correspond with the genetic structure of the subclass. I included a more thorough taxonomic sampling in the present study, in order to produce a more complete picture of the phylogenetic structure present within the subclass.

Several specimens for this chapter were alcohol-preserved, acquired from museum collections. I have described the molecular methods I used for determining sequences from these specimens in Chapter 2. Much of the DNA from these specimens was degraded, and standard PCR amplifications were usually unsuccessful. Using an extended DNA extraction protocol combined with PCR reamplifications using taxon-specific primers, sequences of length 700-1800 bp were determined from specimens that had been preserved up to fifty years.

My phylogenetic analyses indicated that the morphologically distinct groups within the Subclass Octocorallia do not constitute phylogenetically distinct entities. Three phylogenetic clades were present within the subclass, none of which corresponded to the

traditional morphologically based divisions. The Pennatulacea were primarily monophyletic, but the pennatulacean genus *Umbellula* branched with the dimorphic alcyonaceans. The Helioporacea did not appear to be phylogenetically dissimilar from species of the Order Alcyonacea. The morphological character of colonial dimorphism was loosely correlated with phylogenetic structure, but this association was not well-supported. Nearly half of the species in this study, including representatives from all five suborders of the Alcyonacea, clustered together in a large, undifferentiated clade. The low levels of genetic divergence within this clade suggest a rapid radiation of species occurred at some point in the evolutionary history of the Anthozoa.

Chapters 3 and 4 include an examination of the phylogenetic affinities of two species with enigmatic morphologies, *Dactylanthus antarcticus* (representing the Order Ptychodactiaria) and *Dendrobrachia paucispina*. The Ptychodactiaria were originally classified as members of the Order Actiniaria (the anemones), although their musculature, nematocysts and mesenterial structure differ from the Actiniaria. In 1949, separate ordinal distinction for the Ptychodactiaria was established with the argument that the Ptychodactiaria represent a separate evolutionary line. In my sequence analyses, *Dactylanthus* branched within the Actiniaria, most closely with the Tribe Athenaria. The close association of *Dactylanthus* with the Athenaria suggested that the ordinal-level distinction of the Ptychodactiaria was phylogenetically unwarranted.

Dendrobrachia paucispina was originally classified with the Order Antipatharia (the black corals) based on its axial morphology, but was recognized at the time as anomalous compared to the other antipatharians. Subsequent examinations of the polyp morphology suggested *Dendrobrachia* was more closely related to the chrysogorgiid octocorals (Alcyonacea: Holaxonia). Based on my 18S sequence analyses, *Dendrobrachia* was closely affiliated with the Octocorallia. The affinity of *Dendrobrachia* with the Octocorallia rather than the Antipatharia suggests that polyp morphology, not axial morphology, is more

indicative of the evolutionary ancestry of this group. *Dendrobrachia* fell within the clade containing the Family Chrysogorgiidae, but was actually more closely related to species of *Corallium* and *Paragorgia*. Morphological characters that support this association are unclear.

This thesis project represents the most comprehensive molecular phylogenetic analysis of the Class Anthozoa to date. Complete sequences from the nuclear 18S rRNA gene were determined from 58 species, representing all extant orders of the subclasses Hexacorallia, Octocorallia and Ceriantipatharia, and 22 of the 30 families within the Alcyonacea (Subclass Octocorallia). Sequences from 19 species in GenBank were also included in the analyses. Previous molecular studies have addressed phylogenetic relationships within the Class Anthozoa, but they all used limited taxonomic sampling and relatively simple methods of analyses. The more thorough taxonomic sampling of this project helped further clarify a number of the relationships examined in other studies. The use of maximum likelihood techniques and realistic evolutionary models for the analyses of these sequence data yielded results that could be compared statistically to alternate hypotheses suggested in other studies.

This characterization of phylogenetic relationships gives us a greater understanding of the evolutionary progression within the Class Anthozoa. The simple morphologies of the species in this group make it difficult to establish taxonomic divisions based solely on morphological characters. As a result, the traditional taxonomy of this group often depicted 'categories' of species based on similar convergent morphologies rather than evolutionarily relevant associations. This project helped illuminate the morphological characters that may have greater significance in retracing the evolution of the Anthozoa. Anthozoans are significant members of nearly all marine environments, and a better understanding of their evolutionary associations and their diversity will help us better manage these important biological resources.

Future Directions

This thesis project included complete 18S rDNA sequences from a very thorough representation of the species within the Class Anthozoa, and used relevant evolutionary models to analyze them. Although these analyses could clearly demonstrate some aspects of the phylogenetic associations within the Anthozoa, a number of relationships could not be determined from these sequence data. The nuclear 18S gene had sufficient phylogenetic signal to establish the primary divisions within the class, but could not resolve many of the associations among those divisions. Although the Ceriantharia were clearly divergent from the remaining Anthozoa based on these 18S sequences, their precise phylogenetic position remains equivocal. Similarly, the relationship between the orders Corallimorpharia and Scleractinia could not be clearly established with 18S information. Sequence information from additional taxa and different genomic regions will be necessary to further clarify these relationships.

There is grandeur in this view of life, with its several powers,
having been originally breathed into a few forms or into one;
and that, whilst this planet has gone cycling on
according to the fixed law of gravity, from so simple a beginning
endless forms most beautiful and most wonderful
have been, and are being, evolved.

—Charles Darwin
The Origin of Species

Appendix 1

Appendix 1 Full alignment of 18S rDNA sequences for Chapter 3, shown in NEXUS format for PAUP*. Positions removed for analyses:

1-48, 63, 107-113, 136, 171-184, 214-233, 242-264, 299-308, 318, 400, 528-529, 533, 537, 566, 572, 718-724, 782-785, 789, 835-837, 913, 948-949, 1314, 1443-1455, 1483-1484, 1578-1581, 1774-1816, 1829-1887.

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Tripedali ATCCGTACG---TTTACTAC TTGATTAACCGTGT-AATT CTAGAGCTAATACATGCGA-AGAGTCCGACT-----TCTGGAAGGGATGTATTTA
Trichoplia ATCCGTACG---TTTACTAC TTGATTAACCGTGT-AATT CTAGAGCTAATACATGCGA-AGAGTCCGACT-----TCTGGAAGGGATGTATTTA

Anemoniaag TTGATTCACAAACCAATGCG GGT- -C-TGCCCCG TGCT- -TTGATTCATCAGTAAC TGATCGAATCCGATGCGCTT
 Anthopleu TTGATTCACAAACCAATGCG GGT- -C-GGCCCCG TGCG- -TTGATTCATCAGTAAC TGATCGAATCCGATGCGCTT
 Anemonel TTGATTCACAAACCAATGCG GGT- -C-TGCCCCG TTCT- -TTGATTCATCAGTAAC TGATCGAATCCGATGCGCTT
 Lepidistis TTGATTCACAAACCAATGCG GGT- -C-CAGCCCCG T-ACC- -TTGATTCATCAGTAAC TGATCGAATCCGATGCGCTT
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 Bellonell TTGATTCACAAACCAATGCG GGT- -C-AAGCCCCG TTACC- -TTGATTCATCAGTAAC TGATCGAATCCGATGCGCTT
 Protocell TTGATTCACAAACCAATGCG GGT- -C-CAGCCCCG TT- -TTGATTCATCAGTAAC TGATCGAATCCGATGCGCTT
 N_bowersi TTGATTCACAAACCAATGCG GGT- -C-GGCCCCG T- -TTGATTCATCAGTAAC TGATCGAATCCGATGCGCTT
 Stichopat TTGATTCACAAACCAATGCG GGT- -C-CAGCCCCG TG- -TTGATTCATCAGTAAC TGATCGAATCCGATGCGCTT
 Hormathi TTGATTCACAAACCAATGCG GGT- -C-CAGCCCCG TT- -TTGATTCATCAGTAAC TGATCGAATCCGATGCGCTT
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 Dactylant TTGATTCACAAACCAATGCG GGT- -C-CAGCCCCG TT- -TTGATTCATCAGTAAC TGATCGAATCCGATGCGCTT
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 Rhizopsam TTGATTCACAAACCAATGCG GGT- -C-CAGCCCCG TT- -TTGATTCATCAGTAAC TGATCGAATCCGATGCGCTT
 Tubastrae TTGATTCACAAACCAATGCG GGT- -C-CAGCCCCG TT- -TTGATTCATCAGTAAC TGATCGAATCCGATGCGCTT
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 Renilla TTGATTCACAAACCAATGCG GGT- -C-CAGCCCCG TT- -TTGATTCATCAGTAAC TGATCGAATCCGATGCGCTT
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 Mnemiopsi TTGATTCACAAACCAATGCG GGT- -C-CAGCCCCG TT- -TTGATTCATCAGTAAC TGATCGAATCCGATGCGCTT
 Selaginop TTGATTCACAAACCAATGCG GGT- -C-CAGCCCCG TT- -TTGATTCATCAGTAAC TGATCGAATCCGATGCGCTT
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 Tripedali TTGATTCACAAACCAATGCG GGT- -C-CAGCCCCG TT- -TTGATTCATCAGTAAC TGATCGAATCCGATGCGCTT
 Tritoplia TTGATTCACAAACCAATGCG GGT- -C-CAGCCCCG TT- -TTGATTCATCAGTAAC TGATCGAATCCGATGCGCTT

AnemoneiaG -----GCCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTGTGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Anthopleu G-----GCCTGCGGAT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Anemonei -----GAGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Lepidists -----TGCGCGCGCG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Acanthocho CTCTGTGTGCGCGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Bellonelli CTCTGTGTGCGCGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Protophili A-----TGCGCGCGCG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
N.bowersi -----TGCGCGCGCG-AT GATTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Stichopat -----CGAGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Hormathili -----TGCGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Ceratobro -----CGCGCGCGCG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Phyllangl -----GCCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Parazoant TC-----GCCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Enallipsa -----TGCGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Cerianthe TC-----TGCGCGCGCG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Zoanthidi -----TGCGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Corynacti -----TGCGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Dactylant -----TGCGCGCGCG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Haloclava -----TGCGCGCGCGG-AC GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Acanthopt -----TGCTGCGGCG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Metridium -----TGCGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Fungia -----TGCGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Bathypath -----CGAGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Talaroa CTTGTGTGCGCGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Tubipora CA--CTTGTGCGCGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Pavona -----TGCGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Stomphia -----GGCGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Discosoma -----TGAGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Palythoa -----TGCGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Antipathe -----TGAGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Cirrhipath -----TGAGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
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Flomaris -----TGAGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
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Tubastirae -----TGCGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Umbellula -----TGAGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Renilla -----TGAGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Dendro2 -----TGAGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Bilareum -----TGAGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Mnemiopsi -----TGAGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Seliangnop A-----TGAGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Berore -----TGAGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Scypha -----TGAGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Tetilla G-----TGAGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Tripedali -----TGAGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-
Trichopla -----TGAGCTGCGG-AT GTTTCATTCAAAATTTCTGCC CTATCACTGTGCGATGTAA GGTATGGCTTACCATGGTT ACAACGGGTGACGGAGAAAT-

Anemoniag CAGAACTACATACAGG CTTTGT-AGT-CTT-GT -AATTGAAATGATACACT TAAAT-CCITT-AACGAGA TCATTTGAGGCAAGCTG
 Anthopleu CAGAAATACATACAGG CTTTGT-AGT-CTT-GT -AATTGAAATGATACACT TAAAT-CCITT-AACGAGA TCATTTGAGGCAAGCTG
 Anemonei CAGAAATACATACAGG CTTTGT-AGT-CTT-GT -AATTGAAATGATACACT TAAAT-CCITT-AACGAGA TCATTTGAGGCAAGCTG
 Lepidistis CAGAAATACATACAGG CTTTGT-AGT-CTT-GT -AATTGAAATGATACACT TAAAT-CCITT-AACGAGA TCATTTGAGGCAAGCTG
 Acanthocho CAGAAATACATACAGG CTTTGT-AGT-CTT-GT -AATTGAAATGATACACT TAAAT-CCITT-AACGAGA TCATTTGAGGCAAGCTG
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 N_bowersi CAGAAATACATACAGG CTTTGT-AGT-CTT-GT -AATTGAAATGATACACT TAAAT-CCITT-AACGAGA TCATTTGAGGCAAGCTG
 Stichopac CAGAAATACATACAGG CTTTGT-AGT-CTT-GT -AATTGAAATGATACACT TAAAT-CCITT-AACGAGA TCATTTGAGGCAAGCTG
 Hormathi CAGAAATACATACAGG CTTTGT-AGT-CTT-GT -AATTGAAATGATACACT TAAAT-CCITT-AACGAGA TCATTTGAGGCAAGCTG
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 Dactylant CAGAAATACATACAGG CTTTGT-AGT-CTT-GT -AATTGAAATGATACACT TAAAT-CCITT-AACGAGA TCATTTGAGGCAAGCTG
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 Umbellula CAGAAATACATACAGG CTTTGT-AGT-CTT-GT -AATTGAAATGATACACT TAAAT-CCITT-AACGAGA TCATTTGAGGCAAGCTG
 Renilla CAGAAATACATACAGG CTTTGT-AGT-CTT-GT -AATTGAAATGATACACT TAAAT-CCITT-AACGAGA TCATTTGAGGCAAGCTG
 Dendro2 CAGAAATACATACAGG CTTTGT-AGT-CTT-GT -AATTGAAATGATACACT TAAAT-CCITT-AACGAGA TCATTTGAGGCAAGCTG
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 Berce CAGAAATACATACAGG CTTTGT-AGT-CTT-GT -AATTGAAATGATACACT TAAAT-CCITT-AACGAGA TCATTTGAGGCAAGCTG
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 Tetilla CAGAAATACATACAGG CTTTGT-AGT-CTT-GT -AATTGAAATGATACACT TAAAT-CCITT-AACGAGA TCATTTGAGGCAAGCTG
 Tripedali CAGAAATACATACAGG CTTTGT-AGT-CTT-GT -AATTGAAATGATACACT TAAAT-CCITT-AACGAGA TCATTTGAGGCAAGCTG
 Trichopia CAGAAATACATACAGG CTTTGT-AGT-CTT-GT -AATTGAAATGATACACT TAAAT-CCITT-AACGAGA TCATTTGAGGCAAGCTG

Anemone1g GTGCCAGCAGCCGCGG--TA ATTCAGCTCCAAATAGCGTA TATTAAAGTTGTTGCAGTTA AAAAGCTCGTAGTTGAGATT CGGGAT--GGCACGCGCGGTC
 Anthopleu GTGCCAGCAGCCGCGG--TA ATTCAGCTCCAAATAGCGTA TATTAAAGTTGTTGCAGTTA AAAAGCTCGTAGTTGAGATT CGGGAT--GGGCG--CGGTC
 Anemone1 GTGCCAGCAGCCGCGG--TA ATTCAGCTCCAAATAGCGTA TATTAAAGTTGTTGCAGTTA AAAAGCTCGTAGTTGAGATT CGGGAT--GGGCGCGCGCGGTC
 Lepidistis GTGCCAGCAGCCGCGG--TA ATTCAGCTCCAAATAGCGTA TATTAAAGTTGTTGCAGTTA AAAAGCTCGTAGTTGAGATT CGG--AGCGCGCGGTTGTC
 Acanthopo GTGCCAGCAGCCGCGG--TA ATTCAGCTCCAAATAGCGTA TATTAAAGTTGTTGCAGTTA AAAAGCTCGTAGTTGAGATT CGGGAT--GGGCGCGCGCGGTC
 Bellonell GTGCCAGCAGCCGCGG--TA ATTCAGCTCCAAATAGCGTA TATTAAAGTTGTTGCAGTTA AAAAGCTCGTAGTTGAGATT CGG--CTGCAGCGCGGCGGTC
 Protodell GTGCCAGCAGCCGCGG--TA ATTCAGCTCCAAATAGCGTA TATTAAAGTTGTTGCAGTTA AAAAGCTCGTAGTTGAGATT CGGGAT--GGGCGCGGTTGTC
 Stichopat GTGCCAGCAGCCGCGG--TA ATTCAGCTCCAAATAGCGTA TATTAAAGTTGTTGCAGTTA AAAAGCTCGTAGTTGAGATT CGGGAT--GGCACGCGCGGTC
 Hormathi1 GTGCCAGCAGCCGCGG--TA ATTCAGCTCCAAATAGCGTA TATTAAAGTTGTTGCAGTTA AAAAGCTCGTAGTTGAGATT CGGGAT--GGGCGCGCGGTC
 Ceratotre GTGCCAGCAGCCGCGG--TA ATTCAGCTCCAAATAGCGTA TATTAAAGTTGTTGCAGTTA AAAAGCTCGTAGTTGAGATT CGGGAT--GGGCGCGCGGTC
 Phyllangi GTGCCAGCAGCCGCGG--TA ATTCAGCTCCAAATAGCGTA TATTAAAGTTGTTGCAGTTA AAAAGCTCGTAGTTGAGATT CGGGAT--GGGCGCGCGGTC
 Cerianthu GTGCCAGCAGCCGCGG--TA ATTCAGCTCCAAATAGCGTA TATTAAAGTTGTTGCAGTTA AAAAGCTCGTAGTTGAGATT CTGGTA--GCATCGATGTC
 Parazoant GTGCCAGCAGCCGCGG--TA ATTCAGCTCCAAATAGCGTA TATTAAAGTTGTTGCAGTTA AAAAGCTCGTAGTTGAGATT CGGGAT--GGCTTTTCGTC
 Enallopsa GTGCCAGCAGCCGCGG--TA ATTCAGCTCCAAATAGCGTA TATTAAAGTTGTTGCAGTTA AAAAGCTCGTAGTTGAGATT CGGGAT--GGGCGCGCGGTC
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 Cliripath TTAAGGAAATTGACGGAAAG GCACCACCGAGAGTGG -AGC CTG -CGGCTTAATTGACTC AACACGGGAAAACCTCACAG GTCCAGACATAGGAAGATT
 Antipatce TTAAGGAAATTGACGGAAAG GCACCACCGAGAGTGG -AGC -TG -CGGCTTAATTGACTC AACACGGGAAAACCTCACAG GTCCAGACATAGGAAGATT
 Epiactis TTAAGGAAATTGACGGAAAG GCACCACCGAGAGTGG -AGC -TG -CGGCTTAATTGACTC AACACGGGAAAACCTCACAG GTCCAGACATAGGAAGATT
 Flomaris TTAAGGAAATTGACGGAAAG GCACCACCGAGAGTGG -AGC -TG -CGGCTTAATTGACTC AACACGGGAAAACCTCACAG GTCCAGACATAGGAAGATT
 Rhizosam TTAAGGAAATTGACGGAAAG GCACCACCGAGAGTGG -AGC -TG -CGGCTTAATTGACTC AACACGGGAAAACCTCACAG GTCCAGACATAGGAAGATT
 Tubastrae TTAAGGAAATTGACGGAAAG GCACCACCGAGAGTGG -AGC -TG -CGGCTTAATTGACTC AACACGGGAAAACCTCACAG GTCCAGACATAGGAAGATT
 Umbellula TTAAGGAAATTGACGGAAAG GCACCACCGAGAGTGG -AGC CTG -CGGCTTAATTGACTC AACACGGGAAAACCTCACAG GTCCAGACATAGGAAGATT
 Renilla TTAAGGAAATTGACGGAAAG GCACCACCGAGAGTGG -AGC CTG -CGGCTTAATTGACTC AACACGGGAAAACCTCACAG GTCCAGACATAGGAAGATT
 Dendro2 TTAAGGAAATTGACGGAAAG GCACCACCGAGAGTGG -AGC CTG -CGGCTTAATTGACTC AACACGGGAAAACCTCACAG GTCCAGACATAGGAAGATT
 Briareum TTAAGGAAATTGACGGAAAG GCACCACCGAGAGTGG -AGC CTG -CGGCTTAATTGACTC AACACGGGAAAACCTCACAG GTCCAGACATAGGAAGATT
 Kheidiopsi TTAAGGAAATTGACGGAAAG GCACCACCGAGAGTGG -AGC CTG -CGGCTTAATTGACTC AACACGGGAAAACCTCACAG GTCCAGACATAGGAAGATT
 Selaginop TTAAGGAAATTGACGGAAAG GCACCACCGAGAGTGG -AGC CTG -CGGCTTAATTGACTC AACACGGGAAAACCTCACAG GTCCAGACATAGGAAGATT
 Berce TTAAGGAAATTGACGGAAAG GCACCACCGAGAGTGG -AGC CTG -CGGCTTAATTGACTC AACACGGGAAAACCTCACAG GTCCAGACATAGGAAGATT
 Scypha TTAAGGAAATTGACGGAAAG GCACCACCGAGAGTGG -AGC CTG -CGGCTTAATTGACTC AACACGGGAAAACCTCACAG GTCCAGACATAGGAAGATT
 Teciella TTAAGGAAATTGACGGAAAG GCACCACCGAGAGTGG -AGC CTG -CGGCTTAATTGACTC AACACGGGAAAACCTCACAG GTCCAGACATAGGAAGATT
 Tripedali TTAAGGAAATTGACGGAAAG GCACCACCGAGAGTGG -AGC CTG -CGGCTTAATTGACTC AACACGGGAAAACCTCACAG GTCCAGACATAGGAAGATT
 Trichoplia TTAAGGAAATTGACGGAAAG GCACCACCGAGAGTGG -AGC CTG -CGGCTTAATTGACTC AACACGGGAAAACCTCACAG GTCCAGACATAGGAAGATT

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Anemonias CCTTAACCTGCTAAATAGT ACGCCATCCG-GATTGGCA ACTA-----ACTTC TTAGAGGACTGTG-GTGT TT--AACCAAGTCAGAA--
Anthopleu CCTTAACCTGCTAAATAGT ACCTCG-TCCT-GACGGCG GCTA-----ACTTC TTAGAGGACTGTG-GTGT TT--AACCAAGTCAGAA--
Anemonel CCTTAACCTGCTAAATAGT ACAGGATCCC-GATTGGCG GTTG-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Lepidistis CCTGGGCTGCTAAATAGT GGAGGATCTC-GATTCGTC CTG-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Acanthopo CCTTAACCTGCTAAATAGT AGAGATCTC-GATTCGTC CTG-----ACTTC TTAGAGGACTGTG-GTGT TT--AACCAAGTCAGAA--
Bellonell CCTTAACCTGCTAAATAGT AGAGATCTC-GATTCGTC CTG-----ACTTC TTAGAGGACTGTG-GTGT TT--AACCAAGTCAGAA--
Protocli CCTTAACCTGCTAAATAGT GGCGGATTCG-GATTCGTA CTG-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
N_bowersi CCTTAACCTGCTAAATAGT GGAGATCTC-GATTCGTC CACG-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Stichopat CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TT--AACCAAGTCAGAA--
Hornathi CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTG-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Ceratocto CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GTTG-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Phyllangi CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Cerialanthu CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Parazoant CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Enallipsa CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Cerialanth CCTTAACCTGCTAAATAGT GGCGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Zoanthidi CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Corynacti CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Dactylant CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Haloclava CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Acanthopt CCTTAACCTGCTAAATAGT TGCGGATCTC-----GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Metridium CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Funglia CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Bathypath CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Talaroa CCTTAACCTGCTAAATAGT AGAGGATCTC-GATTCGTC CTG-----ACTTC TTAGAGGACTGTG-GTGT TT--AACCAAGTCAGAA--
Tubipora CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Pavona CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Stomphia CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Discosoma CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Palythoa CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Antipathe CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Cirripath CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Antipathe CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Epiactis CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Flosmaris CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Rhizosam CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Tubastrae CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Umbellula CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Renilla CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Dendro2 CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Briareum CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Mnemolopi CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Seliginop CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Beroe CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Scypha CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Tetilla CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Tripedali CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--
Trichopla CCTTAACCTGCTAAATAGT ACAGGATCTC-GATTCGTC GCTA-----ACTTC TTAGAGGACTGTG-GTGT TC--AACCAAGTCAGAA--

197

199

Anemonias ----G-CGGAC--TGCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Anthopleu ----G-CGGAA--TGCCGAAA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Anemonei ----G-CGGAC--TGCCGAAA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Lepidistis ----GACGAGAGT-CGAAAC AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Acanthogo C---GACGAGAGT-C-AAAA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Bellonell C---GACGAGAGT-C-AAAA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Protoptil ----GACGAG-G-GCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
N_bowersi ----GACGAGAG-CTTGAAA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Stichopat ----G-CGGA--TGCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Hornathi ----G-CGAC--TGCCGAAA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Ceratopro ----GCGGAGATTTGCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Phyllangi ----GCCGGA--TGCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Cerianthu ----G-CGACCGCTCCGAAA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Parazoant ----GCAGCGGATTTGCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Enallipsa ----GCC--GGATGCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Cerianthe ----CCGGATGCTGCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Zoanthridi ----GC-GGA--TGTCGAAA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Corynacti ----GTCCGA--TGTTGGGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Dactylant ----GC-GGAC--TGCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Haloclava ----GC-GGAC--TGCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Acanthopt ----GACGAGC--GCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Metridium ----GC-GGAC--TGCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Fungia ----GCCGGA--TGCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Bathypath ----GC-GGA--TGCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Talaroa C---GACGAGC--GTCAAAA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Tubipora C---GCCGAGC--GTCAAAA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Pavona ----GCCGAGT--GCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Stomiosoma ----GC-GGA--TGCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Palythoa ----GC-GGACTGTGCGAAA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Antipathes ----GC-GGAT--GCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Cirripath ----GC-GGAT--GCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Antipathes ----GC-GGAT--GCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Epiactis ----GC-GGAT--GCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Fleomaris ----GC-GGA--TGCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Rhizopseam ----GCCGGA--TGCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Tubastrae ----GCCGAGT--TGCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Umbellula ----GACGAG--GCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Renilla ----GACGAG--GCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Dendro2 ----GACGAG--GCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Briareum ----GACGAGGTC--GAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Mnemiopsis ----CACGCGCCGCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Seligtop GTCCACGCGTTAGCCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Beroe GTCCGCC-----GAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Scypha ----GGGCGATAGC-GAAA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Tetilla ----GCCGCGCGCGATTCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Triptedali ----GCTTANGGTTACCGAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC
Tritichopla GTCCCAATTATC-----GAGA AGTTGTTCAA-ACCTGATCA TTTAGAGGAAGTAAAGTCG TAACAAGGTTCCGTAAGTC AACCTGC

end;

Appendix 2

Appendix 2 Full alignment of 18S rDNA sequences for Chapter 4, shown in NEXUS format for PAUP*. Positions removed for analyses:
189-197, 253, 372, 472-474, 501-502, 509, 597, 762-767, 813, 830-833, 839, 1039, 1362-1380, 1524, 1680-1737, 1756-1805.


```

#NEXUS
[gde4173_0 -- data title]

[Name: Anemoniaceae] Len: 1805 Check: 29AFB375]
[Name: Hormathid] Len: 1805 Check: EB2DEBF]
[Name: Narellanut] Len: 1805 Check: 4B00EC95]
[Name: Lepidistis] Len: 1805 Check: AA2D1174]
[Name: Orestomistis] Len: 1805 Check: BE35E131]
[Name: Umbellula] Len: 1805 Check: 57D787A4]
[Name: Paramuriceid] Len: 1805 Check: 5454CE07]
[Name: Acanthogorgia] Len: 1805 Check: ED14DDA7]
[Name: Talaroa] Len: 1805 Check: E469E350]
[Name: Bellonellaceae] Len: 1805 Check: C898DF4F]
[Name: Protophylum] Len: 1805 Check: 72883BA9]
[Name: Anthomastus] Len: 1805 Check: 4BA4A3A3]
[Name: Narellabow] Len: 1805 Check: 82D412B3]
[Name: Protodendron] Len: 1805 Check: 73C4A2B]
[Name: Tubipora] Len: 1805 Check: 72ACD036]
[Name: Paragorgia] Len: 1805 Check: 581F70C8]
[Name: Lophogorgia] Len: 1805 Check: 7F7912C9]
[Name: Chrysogorgia] Len: 1805 Check: 72C0BC2E]
[Name: Acanthopodium] Len: 1805 Check: 177PP559]
[Name: Clavularia] Len: 1805 Check: F2EA24E8]
[Name: CoralliumCR2] Len: 1805 Check: 58547539]
[Name: Renilla] Len: 1805 Check: 289A5E96]
[Name: Stylactula] Len: 1805 Check: DAFCEB7B]
[Name: Virgularia] Len: 1805 Check: EC7367FB]
[Name: LeiopodiumGEN] Len: 1805 Check: 6C06D191]
[Name: AlcyoniumGEN] Len: 1805 Check: 1B9F806F]
[Name: C. Kisch1] Len: 1805 Check: A5A0DE58]
[Name: Lemnalia] Len: 1805 Check: FF4B1823]
[Name: Xenia] Len: 1805 Check: 8D567117]
[Name: Calicogorgia] Len: 1805 Check: E1682F6C]
[Name: Euplexauraceae] Len: 1805 Check: 6AB1783C]
[Name: Anthopodium] Len: 1805 Check: C0B06062]
[Name: Eunicea] Len: 1805 Check: 6D6AE5C4]
[Name: Anthothela] Len: 1805 Check: C6B7B6C]
[Name: Telestula] Len: 1805 Check: DE4ED1C]
[Name: Briareum] Len: 1805 Check: 61061037]
[Name: Siphonogorg] Len: 1805 Check: DDF5A19A]
[Name: Melitella] Len: 1805 Check: FF94E4FD]
[Name: Calcigorgia] Len: 1805 Check: 173781A1]
[Name: Junces2] Len: 1805 Check: 44D4F6EC]
[Name: Coelogorgia] Len: 1805 Check: 12EC02B1]
[Name: Erythropodium] Len: 1805 Check: 94368F93]
[Name: Junces1AT] Len: 1805 Check: EBD040D4]
[Name: Helip2] Len: 1805 Check: 676FED1]
[Name: Plumigorgia] Len: 1805 Check: B961BC4]
[Name: Dendro2] Len: 1805 Check: 7337375]
[Name: Selaginopsis] Len: 1805 Check: A556CC1]
[Name: Berce] Len: 1805 Check: 7B881AE0]
[Name: Mnemiopsis] Len: 1805 Check: EB1875E]
[Name: Scypha] Len: 1805 Check: B159093F]
[Name: Tridacalia] Len: 1805 Check: 2B3B361]
[Name: Trichoplax] Len: 1805 Check: 3B4E51F]
[Name: Tetilla] Len: 1805 Check: 33E53401]

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calycalyptra-intervallae

MATHEMATICS

memoria

[illegible]

205

207

208

Anemone1g AGCTCGTAGTGGACTTTC GGGT--CGACGCGCGGATTC G-CGCA--GTGTCTACTG GCCGAGCCGCTCTCTTCGc MAAGACCGGATCTCTTCG
Hornetballi AGCTCGTAGTGGACTTTC GGA--CGGCGCGCGGATTC G-CGCAAGGATCTCTTCG ACCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Narellianu AGCTCGTAGTGGACTTTC GGA--CGGATCGGATCTTCG G-CGCAAGGATCTCTTCG GCCGACATGTCTCTTCGc MAAGCTGCAATCTCTTCG
Lepidistis AGCTCGTAGTGGACTTTC GAG--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Orzomisti AGCTCGTAGTGGACTTTC GAG--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Umbellula AGCTCGTAGTGGACTTTC GAGT--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Paramarico AGCTCGTAGTGGACTTTC GAGT--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Acanthopio AGCTCGTAGTGGACTTTC GAGT--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Talaroa AGCTCGTAGTGGACTTTC GAGT--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Bellonella AGCTCGTAGTGGACTTTC G-CT--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Protopell AGCTCGTAGTGGACTTTC GAGT--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Anthomast AGCTCGTAGTGGACTTTC GAGT--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Narellabo AGCTCGTAGTGGACTTTC GGA--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Proctend AGCTCGTAGTGGACTTTC GAGT--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Thibpora AGCTCGTAGTGGACTTTC GAGT--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Paragorgi AGCTCGTAGTGGACTTTC GGA--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Lophogorg AGCTCGTAGTGGACTTTC GAGT--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Chysogor AGCTCGTAGTGGACTTTC GGA--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Acanthopt AGCTCGTAGTGGACTTTC GGA--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Clavulati AGCTCGTAGTGGACTTTC GAGT--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Corallium AGCTCGTAGTGGACTTTC GGA--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Renilla AGCTCGTAGTGGACTTTC GAGT--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
Sylatula AGCTCGTAGTGGACTTTC GGA--CGGCGCGGATTC G-CGCAAGGATCTCTTCG GCCGAGCTGTCTCTTCGc MAAGCTGCAATCTCTTCG
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 Lepidasis TGAAC
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			14.
15. Supplementary Notes This thesis should be cited as: Ewann Agenbroad Berntson, 1998. Evolutionary Patterns Within the Anthozoa (Phylum Cnidaria) Reflected in Ribosomal Gene Sequences. Ph.D. Thesis. MIT/WHOI, 98-24.			
16. Abstract (Limit: 200 words) This project assessed phylogenetic relationships within the Class Anthozoa (Cnidaria) using 18S rDNA sequences from 71 taxa, including museum specimens, providing the most thorough taxonomic sampling to date. Morphological taxonomists disagree over the evolutionary relationships within the Anthozoa. Sequence information and relevant evolutionary models were used to address subclass- and ordinal-level divisions within the Anthozoa. Nuclear 18S sequence information did not support some of the traditional classifications within the Anthozoa. Members of the Subclass Ceriantipatharia were genetically disparate, and phylogenetic divisions within the Anthozoa resembled the previous two-subclass system. The orders within the Hexacorallia constituted monophyletic groups with the exception of the Corallimorpharia, which were closely associated with the Scleractinia. The morphological characters of mesentery and tentacle structure were reflected in genetic structure of the Anthozoa. The morphological characters of skeletal structure and colony morphology were not evident in the phylogenetic divisions of the Octocorallia. The current orders and suborders were not monophyletic entities. Colony dimorphism corresponded loosely with genetic divisions within the Octocorallia. Half of the octocoral species included in this study formed a large, undifferentiated group, suggesting that a rapid radiation occurred. This project provides valuable insight into the evolution and diversity of these important organisms.			
17. Document Analysis a. Descriptors 18S rDNA Maximum likelihood Octocorallia b. Identifiers/Open-Ended Terms c. COSATI Field/Group			
18. Availability Statement Approved for publication; distribution unlimited.		19. Security Class (This Report) UNCLASSIFIED	21. No. of Pages 222
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